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## EFFECT OF DIOXIN ON BONE CELL PROTEOME

Thesis submitted on 29<sup>th</sup> September 2008 by

**Donatella CARPI**

For the degree of Doctor of Philosophy

Istituto di Ricerche Farmacologiche "Mario Negri" Milano  
Via La Masa 19, 20156, Milan, Italy  
Tel.: +39-02-39014518  
Fax.: +30-02-354-6277  
*e-mail:* carpi@marionegri.it

**The Open University, UK**

— Advanced School of Pharmacology —  
Dean, Enrico Gurattini M D

Mario Negri Institute for  
Pharmacological Research

17/3/2008

Director of Studies: Dr. Roberta Pastorelli  
Protein and Gene Biomarkers Unit  
Laboratory of Molecular Toxicology  
Department of Environmental Health Sciences  
Istituto di Ricerche Farmacologiche "Mario Negri" Milano  
Via La Masa 19, 20156 Milan, Italy  
Tel.: +39-02-39014397 Fax.: +30-02-354-6277  
*e-mail:* [rpastorelli@marionegri.it](mailto:rpastorelli@marionegri.it)

Supervisor: Dr. Robin Wait  
Dr Robin Wait,  
Senior Lecturer in Proteomics  
Kennedy Institute of Rheumatology Division  
Imperial College London  
1 Aspenlea Road, Hammersmith, London, W6 8LH,  
Tel: +44 (0)20 8383 4454 Fax: +44 (0) 20 8383 4499  
*e-mail:* [r.wait@imperial.ac.uk](mailto:r.wait@imperial.ac.uk)

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## ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an endocrine disrupting environmental pollutant that affects bone tissue, although the mechanistic basis of such effect is far from clear.

In this study a proteomic approach has been adopted to investigate the disturbance of the osteogenic process evoked by TCDD in an *in vitro* osteoblast differentiation model of rat mesenchymal stem cells. Stem cells were isolated from bone marrow of femurs and tibias of rats. Progress of osteoblastic differentiation was monitored by measuring mRNA expression levels of differentiation markers from control and TCDD-treated cells after 3, 7 and 10 days of culture in presence and absence of TCDD using quantitative RT-PCR.

Analysis of differential protein expression in the total cell lysate of untreated and TCDD-treated cells was carried out over the same time points, using two-dimensional gel electrophoresis, computerized gel image, univariate and multivariate statistical analysis, in-gel digestion and tandem mass spectrometry for protein identification.

Expression of all measured markers characteristic for various stages of osteoblastic differentiation (Runx2, alkaline phosphatase and osteocalcin) was dramatically reduced only after 10 days of TCDD exposure, indicating that TCDD significantly inhibits osteoblast differentiation *in vitro*.

Similarly, the most significant rearrangement of the proteome during osteoblast differentiation was observed following the 10 days of TCDD exposure, at which time the full progress of osteoblast differentiation should have occurred. Overall, 18 individual proteins showed a statistically significant change in abundance as a result of 10 days of TCDD exposure,

These proteins were mostly involved in cytoskeleton organization and biogenesis, actin filament-based processes, protein transport and folding. The alteration in cell

architecture and increase in cell adhesion were confirmed by confocal microscopy. The TCDD-induced decrease in the expression of calcium binding proteins may interfere with osteoblast calcium deposition, which was in fact reduced by TCDD. Evidence from proteomics and Western blot analysis indicated a decreased apoptotic capability in TCDD-treated osteoblasts.

This is the first study investigating, at the protein expression level, the effect evoked by TCDD during osteoblastic differentiation. Interestingly, MetaCore pathway analysis grouped the majority of these proteins around two principal nodes (*c-fos* and *c-myc*) suggesting that they may participate in the transcriptional activation of key pathways in TCDD-driven inhibition of osteoblast differentiation. These findings provide clues to the complex interplay between TCDD's action and the regulatory molecules that alter signal-transduction pathways regulating osteoblast differentiation and indicate new molecular players in the effects of TCDD on bone development.

# CONTENTS

ACKNOWLEDGEMENTS.....	2
ABSTRACT.....	3
CONTENTS.....	5
LIST OF FIGURES .....	9
LIST OF TABLES .....	13
<b>CHAPTER 1. INTRODUCTION .....</b>	<b>14</b>
1.1. Dioxins .....	15
1.1.1. Chemical and physical characteristics .....	15
1.1.2. Dioxins: environmental distribution .....	16
1.1.3. Toxicological effects .....	18
1.1.4. Mechanisms of action .....	21
1.1.5. TCDD as endocrine disruptor .....	25
1.2. General aspects on bone .....	27
1.2.1. Bone tissue macroscopic organization: anatomy and physiology .....	27
1.2.2. Bone cells and bone matrix composition .....	29
1.2.3. Bone remodelling .....	33
1.2.4. Bone remodelling and systemic hormones .....	36
1.3. Osteoblast lineage .....	38
1.3.1. Mesenchymal stem cells.....	38
1.3.2. Osteoblast differentiation process .....	39
1.4. Dioxin and bone .....	46
1.5. Proteomics .....	48
1.5.1. Proteomics in the field of toxicology: toxicoproteomics .....	48
1.5.2. Introduction to proteomic analysis.....	49
1.6. Protein identification by Mass-Spectrometry .....	54

1.6.1. Principles of Mass Spectrometry.....	54
1.6.2. Protein cleavage.....	56
1.6.3. Sample introduction into the mass spectrometer .....	57
1.6.4. Ion sources.....	58
1.6.5. Mass analysers.....	60
1.6.6. Tandem Mass-Spectrometry significance .....	63
1.6.7. Protein identification and database searching.....	65
1.7. Pathway analysis .....	67
<b>CHAPTER 2. AIMS OF THE THESIS.....</b>	<b>68</b>
<b>CHAPTER 3. MATERIALS AND METHODS.....</b>	<b>71</b>
3.1. Reagents.....	72
3.2. Mesenchymal stem cell isolation and osteoblast differentiation.....	72
3.3. Dioxin exposure.....	74
3.4. Sample collection.....	75
3.5. Cell proliferation and calcium quantification assay.....	76
3.6. Real time Quantitative PCR.....	77
3.7. Protein extraction for 2D-Gel electrophoresis.....	79
3.7.1. Set-up of protein extraction protocol .....	79
3.7.2. Application of the optimized protein extraction protocol .....	82
3.8. Protein quantification for two-dimensional gel electrophoresis .....	83
3.9. Two-dimensional gel electrophoresis (2-DE).....	84
3.10. Two-dimensional gel electrophoresis gel image analysis .....	86
3.10.1. Progenesis PG240 analysis.....	86
3.10.2. Progenesis SameSpots analysis.....	87
3.11. Proteomic statistical analysis .....	88
3.11.1. Univariate statistical approach .....	88
3.11.2. Multivariate statistical approach .....	89

3.12. Protein identification by tandem mass spectrometry.....	90
3.12.1. Manual protein in-gel digestion .....	90
3.12.2. Robotic protein in-gel digestion.....	91
3.12.3. Liquid chromatography-ESI (electrospray ionization)- ion trap tandem mass spectrometry.....	92
3.12.4. Liquid chromatography-ESI (electrospray ionization)-Quadrupole-Time of Flight mass spectrometry.....	94
3.12.5. Liquid chromatography-MALDI (matrix assisted laser desorption ionization)-quadrupole-Time of Flight mass spectrometry .....	95
3.13. Western Blot Analysis .....	96
3.13.1. Protein extraction for Western blot analysis .....	96
3.13.2. Protein quantification for Western blot analysis .....	96
3.13.3. Western Blot analysis.....	96
3.14. Confocal microscopy.....	98
3.15. Pathways analysis .....	99
<b>CHAPTER 4. RESULTS.....</b>	<b>100</b>
4.1. Effect of TCDD on cell growth and differentiation .....	101
4.1.1. Cell proliferation and vitality .....	101
4.1.2. Calcium deposition .....	102
4.1.3. Effect of TCDD on cell growth and differentiation: remarks .....	103
4.2. Effect of TCDD on time-dependent expression of osteogenic markers.....	104
4.2.1 Effect of TCDD on time-dependent expression of osteogenic markers: remarks .....	104
4.3. Differential proteomic analysis using univariate approach .....	106
4.3.1. Proteome profile of differentiating osteoblasts exposed for 10 days to TCDD .....	107

4.3.2. Proteome profile of differentiating osteoblasts exposed to TCDD at earlier time points.....	119
4.3.3. Time-course analysis of the identified proteins susceptible to TCDD in relation to the progress of osteoblastic differentiation .....	121
4.3.4. Differential proteomic analysis using univariate approach: remarks.....	125
4.4. Differential proteomic analysis using a multivariate approach for data assessment.....	129
4.4.1. Differential proteomic analysis using a multivariate approach for data assessment: remarks .....	143
4.5. Confocal microscopy .....	145
4.5.1. Confocal microscopy: remarks .....	147
4.6. Pathway analysis .....	148
4.6.1. Functional ontology enrichment .....	148
4.6.2. Biological Network analysis.....	149
4.6.3. Pathway analysis: remarks .....	150
<b>CHAPTER 5. CONCLUDING DISCUSSION .....</b>	<b>154</b>
<b>CHAPTER 6. REFERENCES .....</b>	<b>165</b>
<b>CHAPTER 7. APPENDIX .....</b>	<b>197</b>
7.1. List of abbreviations.....	198
7.2. List of publications .....	200
7.3. Supplemental tables 1 and 2.....	202

## LIST OF FIGURES

<b>Figure 1.</b> Halogenated aromatic hydrocarbon basic molecular structure.	51
<b>Figure 2.</b> Aryl hydrocarbon receptor molecular mechanism.	22
<b>Figure 3.</b> Stress-activated kinase pathways.	24
<b>Figure 4.</b> Schematic view of a long bone and its organization.	28
<b>Figure 5.</b> Schematic representation of the communication between osteoblasts and osteoclasts during bone remodelling.	35
<b>Figure 6.</b> Stem cell commitment to mesenchymal phenotypes.	39
<b>Figure 7.</b> Growth and differentiation of osteoblasts.	40
<b>Figure 8.</b> Regulation of transcription factors during osteoblastogenesis.	44
<b>Figure 9.</b> Proteomic workflow.	49
<b>Figure 10.</b> A schematic indicating the main regions of a mass spectrometer.	54
<b>Figure 11.</b> Images of the two mass spectrometers utilised for protein identification in this thesis.	55
<b>Figure 12.</b> Schematic of ESI.	59
<b>Figure 13.</b> Operating Principle of a Quadrupole Mass Spectrometer.	61
<b>Figure 14.</b> A schematic of a Quadrupole Ion Trap Mass Analyser.	61
<b>Figure 15.</b> A schematic of a Time-of-Flight mass spectrometer operating in Reflectron Mode.	62
<b>Figure 16.</b> Fragmentation nomenclature.	64
<b>Figure 17.</b> Mesenchymal stem cells (MSCs) isolation.	73
<b>Figure 18.</b> Experimental design.	74
<b>Figure 19.</b> Colloidal Coomassie Blue-stained 2-DE gels of whole cell lysate obtained using different extraction buffers.	81
<b>Figure 20.</b> Effect of TCDD on time-dependent cell proliferation of differentiating osteoblasts.	102

<b>Figure 21.</b> Effect of 10 days of TCDD exposure on osteoblasts calcium deposition.	102
<b>Figure 22.</b> Effect of TCDD on time-dependent mRNA expression of Runx2, Alkaline Phosphatase (Alp) and Osteocalcin (Ocn) in differentiating osteoblasts.	105
<b>Figure 23.</b> Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate from unexposed osteoblasts (DMSO). Protein species with lower (down) or greater (up) expression in 10 days TCDD exposed osteoblasts compared to unexposed osteoblasts are indicated.	108
<b>Figure 24.</b> Overview of the expression patterns of the 27 protein species, whose abundance changed significantly in differentiating osteoblasts after ten days of TCDD exposure.	111
<b>Figure 25.</b> Rat vimentin aminoacid sequence and graphic representation of the peptide coverage found for each vimentin spot identified by MS/MS.	114
<b>Figure 26.</b> Contribution of each LMNA isoform to the sum of the isoforms in osteoblasts exposed or not to 1nM and 100nM TCDD.	115
<b>Figure 27.</b> Changes in ANXA2, CALR, LMNA, VIM, BAX and BCL2 protein levels in differentiating osteoblasts untreated (DMSO) or after 10 days of exposure to 1nM and 100nM TCDD analyzed by Western blot.	118
<b>Figure 28.</b> Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate from unexposed osteoblasts (DMSO). The protein spot, whose abundance is significant decreased by 7-days of TCDD exposure is highlighted.	119
<b>Figure 29.</b> Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate (200 µg proteins) from unexposed osteoblasts (DMSO) at three days of cell culture.	120
<b>Figure 30.</b> Expression patterns of the identified protein species susceptible to TCDD effect, in relation to the progress of osteoblastic differentiation.	123



<b>Figure 31.</b> Expression levels for the selected proteins whose patterns of expression accompanying the differentiation progress were reverted by 10 days of TCDD exposure.	124
<b>Figure 32.</b> PCA score and loadings plot of the two first PCs of the whole dataset.	130
<b>Figure 33.</b> PCA score and loadings plot of the two first PCs of the dataset referring to untreated samples.	131
<b>Figure 34.</b> Discriminating spots location of 2-DE gel image, dendrogram tree where similar protein expression profiles cluster together and the corresponding expression profiles.	132
<b>Figure 35.</b> PCA score and loadings plot of the two first PCs of the dataset referring to untreated samples and discriminating spots location of 2-DE gel image.	137
<b>Figure 36.</b> PCA score and loadings plot of the two first PCs of the dataset referring to 3-days untreated and TCDD treated samples.	138
<b>Figure 37.</b> PCA score and loadings plot of the two first PCs of the dataset referring to 7-days untreated and TCDD treated samples.	139
<b>Figure 38.</b> PCA score and loadings plot of the two first PCs of the dataset referring to 10-days untreated and TCDD treated samples.	139
<b>Figure 39.</b> Location on the 2-DE gel image of the 42 discriminating spots for the PCA analysis of 10-days exposure untreated <i>vs.</i> TCDD 100nM treated samples.	140
<b>Figure 40.</b> Expression profile cluster of the 42 discriminating spots for the PCA analysis of 10-days exposure untreated <i>vs.</i> TCDD 100nM (TCDD2) treated samples.	141
<b>Figure 41.</b> Venn diagram of osteoblast proteins expressed differentially after	

10 days of TCDD exposure using two statistical approaches. In red are proteins with FDR<5% derived from multivariate analysis.	143
<b>Figure 42.</b> Effects of TCDD on focal adhesion complexes.	147
<b>Figure 43.</b> Biological processes associated with differently expressed proteins in unexposed and 10 days TCDD-exposed differentiating osteoblasts.	150
<b>Figure 44.</b> Biological network analysis of differently expressed proteins in differentiating osteoblasts in response to 10 days of TCDD exposure (univariate analysis).	152
<b>Figure 45.</b> Biological network analysis of differently expressed proteins in differentiating osteoblasts in response to 10 days of TCDD exposure (multivariate analysis).	153
<b>Figure 46.</b> Transcription regulation networks.	154

## LIST OF TABLES

<b>Table 1.</b> Main dioxin emission sources, on the basis of European data.	16
<b>Table 2.</b> AHRE-I and AHRE-II sequence.	23
<b>Table 3.</b> Composition of different lysis buffers used in protein extraction.	80
<b>Table 4.</b> Submission parameters to Phenyx MS/MS search engine.	94
<b>Table 5.</b> Identification of proteins showing different levels of expression in untreated and 10 days TCDD-treated rat osteoblasts, by 2 different LC-MS/MS platforms.	109
<b>Table 6.</b> Identification of proteins showing different levels of expression characterizing untreated osteoblast proteome profile at different time points, by 2 different LC-MS/MS platforms.	134
<b>Table 7.</b> Protein function.	142
<b>Supplemental Table 1-2.</b> Detailed information on peptide/protein identification.	202

**CHAPTER 1**

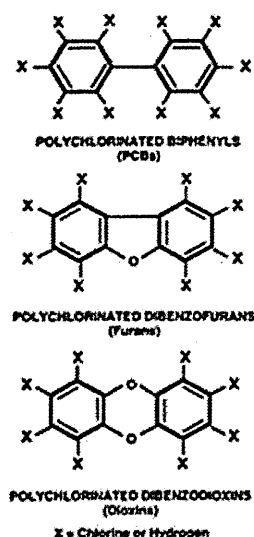
**INTRODUCTION**

## 1.1. Dioxins

### 1.1.1. Chemical and physical characteristics

Dioxins (polychlorinated dibenzo-*p*-dioxins) are ubiquitous and persistent environmental contaminants. The term dioxin-like is commonly used to refer to a group of 30 halogenated aromatic hydrocarbon that share a similar chemical structure and a common mechanism of toxic action. These compounds are members of three closely related families: 7 of the 75 congeners of polychlorinated dibenzo-*p*-dioxins (CDDs), 10 of the 135 possible congeners of chlorinated dibenzofurans (CDFs) and 13 of 209 polychlorinated biphenyls (PCBs) congeners are thought to have dioxin-like toxicity. Their basic molecular structure is shown in **figure 1**[1].

**Figure 1.** Halogenated aromatic hydrocarbon basic molecular structure.



Hydrogen atoms attached to the carbon atoms may be substituted with a chlorine atom. The physical/chemical properties of each congener vary according to the degree and position of chlorine substitution.

Dioxins are solids with high melting and boiling points. There is some variation, however, between the volatility of individual congeners. Dioxins are almost insoluble in

water, but are quite soluble in organic solvents and fats and thus tend to bioaccumulate in tissue lipids and food chain.

**Dioxins: environmental distribution**

**1.1.2.1. Emission sources**

Dioxins are unintentional byproducts of thermal processes and of chemical formulations. The main dioxin emission sources are listed in **Table 1**, on the basis of European data [2].

**Table 1.** Most important dioxin and furan air emission sources.

Source type	PCDD/PCDF (g I-TEQ/year)	Emissions	Uncertainty of EF/AR
MSW incineration	1,437–174	Decreasing trend <sup>a</sup>	Low/low
Sinter plants <sup>b</sup>	1,010–115		Medium/low
Residential wood combustion	945	Extent of contaminated wood used uncertain	
Clinical waste incineration	816	Few plant data and statistics	High/high
Wood preservation	381	From PCP-treated goods	V.high/v.high
Fires	380		V.high/v.high
Non-ferrous metals	136	Cu, Al, Zn	Medium/low
Road transport	111	Mainly leaded fuel; decreasing trend	Low/low
Total	5545		

<sup>a</sup> Illegal domestic burning of MSW  
<sup>b</sup> Sinter plant for recycled materials

*I-TEQ* international toxic equivalent, *EF* emission factor, *AR* activity rate, *MSW* municipal solid waste.

Two major categories of sources can be distinguished [3]: (i) Formation during incineration processes at low temperature (It has been discovered that above 800 °C dioxins are destroyed). This includes municipal waste combustion, scrap metal recycling, vehicle fuel combustion, cigarette smoking, and combustion of wood. (ii) Formation as by-products occurs in industrial processes, such as in the production of pesticides and herbicide and in the pulp and paper industry. Dioxin emissions from

combustion and industrial sources can potentially be explained by three principal mechanisms that should not be regarded as being mutually exclusive. In the first mechanism (referred to as "pass through"), dioxins are present as contaminants in the combusted organic material; they pass through the furnace and are emitted unaltered. In the second mechanism (referred to as "precursor"), dioxins ultimately form from the thermal breakdown and molecular rearrangement of precursor ring compounds, which are defined as chlorinated aromatic hydrocarbons that have a structural resemblance to the dioxins molecules. Ringed precursors that emanate from the combustion zone are a result of the incomplete oxidation of the constituents of the feed (*i.e.*, products of incomplete combustion). The third mechanism (referred to as "*de novo* synthesis") is similar to the precursor mechanism. *De novo* synthesis describes a pathway of dioxins formation from heterogeneous reactions on fly ash (particulate matter) involving carbon, oxygen, hydrogen, chlorine, and a transition metal catalyst. With these reactions, intermediate compounds that have an aromatic ring structure are formed. Moreover, mobilization of dioxins from secondary sources, such as waste dumps and the application of sewage sludge for fertilization, may occur.

#### *1.1.2.2. Human exposure sources*

Dioxins are highly persistent in the environment and are ubiquitously present as mixtures in soil, sediments and air. Due to their lipophilicity, dioxins tend to accumulate in the fat, having a high biological half-life and consequently they bio-magnify in the food chain. Over 90 % of human background exposure to dioxins occurs through the diet, from animal origin [4]. Moreover occupational (*e.g.* as a result of production and use of chlorophenols and chlorophenoxy herbicides) and accidental (Seveso 1976 *e.g.*) exposure to dioxins at higher levels have been reported [5].

### ***Toxicological effects***

Dioxin causes a wide array of adverse health effects in both animals and humans. The wide range of health effects of dioxins are species- and sex-dependent. The type of exposure (acute vs. chronic) and the amount of exposure are also important determinants of the ultimate toxic effects dioxin exposure [6].

2,3,7,8-tetrachloro-p-dioxin (TCDD) is the most toxic dioxin congener and is the prototypical representative of this class of persistent environmental contaminants [7].

Since dioxins differ in their toxic potential, the toxic equivalence factor (TEF) of each dioxin congener is evaluated in relation to the most toxic one (TCDD), so the toxicity of a mixture can be expressed in terms of its Toxic Equivalents (TEQ), which is the amount of TCDD it would take to equal the combined toxic effect of all the dioxins found in that mixture [8].

#### ***1.1.3.1. Toxicological effects on humans and risk assessment***

Short-term exposure of humans to high levels of dioxins occurred during accidental exposure may result in skin lesions, such as chloracne and patchy darkening of the skin, and altered liver function [9]. Long-term exposure is linked to impairment of the immune system, of nervous system development, of the endocrine system and reproductive functions [10]. TCDD is also considered to be a "known human carcinogen" [2, 8, 11].

Although human intake levels of TCDD have decreased notably over the past two decades, a significant baseline exposure to dioxin appears to be inevitable and there are some subgroups in the population that are exposed to higher intakes of dioxins [12, 13].

In the last few years the risk assessment for dioxins and dioxin-like (DL) compounds has been revised. The Scientific Committee on Food of the European Commission (EC



SCF) updated its opinion on the risk assessment of PCDDs, PCDFs and DL-PCBs and adopted a tolerable weekly intake (TWI) of 14 pg TEQ<sub>(1998)</sub> PCDDs/Fs+DL-PCBs kg-bw<sup>-1</sup> [14], whereas the joint FAO/WHO [15]. Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Monthly Intake (PTMI) of 70 pg WHO-TEQ<sub>(1998)</sub> PCDDs/Fs + DL-PCBs kg-bw<sup>-1</sup>.

#### *1.1.3.2. Toxicological effects on animal models*

Dioxin causes a wide range of toxic effects on laboratory animals [6]. These include:

##### *Wasting Syndrome*

Dioxins are some of the most anorexogenic compounds known. Wasting is the main cause of death in laboratory animals exposed to high doses of dioxin-like compounds. This wasting syndrome mainly results from hypophagia, but the exact mechanisms of wasting are still unknown [16, 17]. Significant studies have suggested a causative relationship between increased levels of plasma free tryptophan and increased brain serotonin synthesis, thereby suppressing food intake has been proposed.

##### *Interference with vitamin A system*

Decreased hepatic vitamin A stores occur at exposure levels as low as 14 ng TCDD/kg/day in rats [18, 19] levels at which very few other measurable effects of dioxin exposure can be observed. Other effects on the vitamin A system include altered whole-body turnover of retinoids [20].

### *Immunosuppression*

Immunosuppression is described as one of the most sensitive effects of organochlorine exposure. Juvenile rats fed a few nanograms of TCDD /kg body weight/day for 25 days displayed a significant impaired immune system, including reduced thymic cellularity and splenocyte functionality and lower populations of circulating mature T-lymphocytes [21].

### *Cancer*

Examples of the cell-cycle-toxic/tumorigenic effect of TCDD include the ability to decrease levels of cellular growth factors such as TGFbeta [22], to create chromosomal instability through indirect suppression of the mitotic checkpoint protein MAD2 [23] and to promote direct protein-protein interactions between the AHR and retinoblastoma (Rb) protein that controls cell cycle progression through G1 phase [24]. Interestingly recent findings in AHR over-expressing mice suggest that chronic AHR activation may lead to stomach tumors [25]. Thus, total absence of AHR activation as well as chronic inappropriate activation, may both promote carcinogenesis.

### *Diabetes*

The role of dioxin in type 2 diabetes has been widely studied in Vietnam war veterans exposed to dioxins via the AGENT Orange. Veterans with high blood levels of TCDD had greater prevalence of diabetes and were more likely to be hyperinsulinemic [26]. The molecular mechanism(s) for TCDD interference with insulin action is not well defined, but involves the PPAR/RXR signalling pathway [27].

*Disturbance in the CNS*

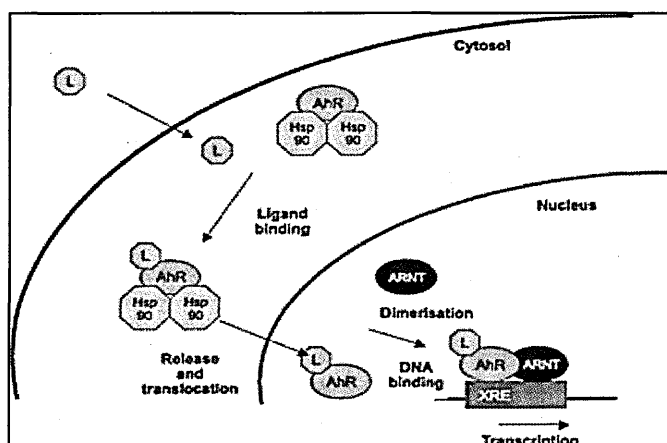
TCDD has well-known adverse effects on the brain and neuronal functions. Specific targets for adverse effects of dioxins on the CNS include dopamine synthesis and signaling, serotonin metabolism, cholinergic nicotine receptor levels and tryptophan levels [28, 29].

*Adverse effects on male and female reproduction*

TCDD has adverse effects on male reproduction system, resulting in decreased weights of the testis, epididymis, seminal vesicle as well as in decreased sperm production [30]. Anti-androgenic effects of dioxins identified at molecular level include altered levels, metabolism, and signalling of testosterone as well as estrogen [31]. Recently, a direct physical interaction between liganded AHR and estrogen receptor ER (both alpha and beta isoforms) was shown, which enhanced ER signalling [32]. TCDD has anti-estrogenic effects in females. Dioxin inhibits the action of estrogen in females of several species and disrupts normal estrous cycling. Other indicators of its antiestrogenic actions are uterine atrophy and decreased number of corpora lutea. Many studies have demonstrated the existence of inhibitory AHR-estrogen receptor (ER) cross-talk in rodent uteri and mammary glands.

**1.1.4. Mechanisms of action**

The molecular mechanisms underlying the multifaceted toxicity of TCDD are still largely unknown, although it has been shown that many of its biochemical and toxic effects are mediated by the aryl hydrocarbon receptor AHR (**figure 2**) [33].



**Figure 2.** Aryl Hydrocarbon receptor molecular mechanism.

#### *1.1.4.1. Mechanism of action via AHR*

The aryl hydrocarbon receptor (AHR) is a member of the basic helix–loop–helix (bHLH)-Per-ARNT-Sim (PAS) family of transcriptional regulators and is highly conserved among the mammalian species. Unliganded inactive AHR is located in the cytoplasm associated with heat shock protein 90 (HSP90) and a 38 kDa, immunophilin-related protein (XAP2) and some other auxiliary chaperones [34]. Ligand binding to the AHR is presumed to produce conformational changes in the AHR protein, which results in the exposure of an AHR nuclear localization signal and the translocation of the whole complex into the nucleus. Within the nucleus, the AHR–ligand complex dissociates from associated proteins and dimerizes with ARNT to reconstitute an active transcription factor, which binds with high affinity to a specific DNA sequence, known as aryl hydrocarbon response element I (AHRE-I) or xenobiotic-responsive element (XRE), or dioxin-responsive element (DRE). This sequence is located in regulatory regions of aryl hydrocarbon-responsive genes (**table 2**, AHRE-I sequence). Binding to the AHRE-I activates the transcription of a battery of target genes, the most known of which is the cytochrome P450 1A1 (CYP1A1) [35, 36].

The aryl hydrocarbon receptor repressor (AHRR) is a negative regulator of AHR, which acts by competing with AHR for dimerization of ARNT and for binding to the AHRE-I sequence. In addition, AHRR is inducible by AHR ligands and thus regulates AHR function by a negative feedback mechanism affecting the expression of genes induced by dioxins [37, 38].

Recently, it has been proposed that the AHR-ARNT heterodimer may bind to another response element called AHRE-II (**table 2**, AHRE -II sequence) while associated with an unidentified factor [39]. Binding of this complex appears to lead to the activation of a novel, functionally coherent battery of genes [40, 41].

**Table 2.** Response element site sequences.

Regulatory element name	Sequence
AHRE-I (core)	GCGTG
AHRE-I (extended)	TNGCGTG
AHRE-I (full)	(T/G)NGCGTG(A/C)(G/C)A
AHRE-II CATGN6C(T/A)TG	CATGN6C(T/A)TG
ARE TGACNNNGC	TGACNNNGC

Exposure to TCDD and subsequent recruitment of ARNT through AHR may inhibit further signal transduction pathways depending on ARNT [42]. Thus, through its ability to interact with multiple signal transduction pathways and to induce or inhibit a variety of gene products, AHR agonists are capable of inducing a wide spectrum of biological effects at a number of different life stages and in a variety of species.

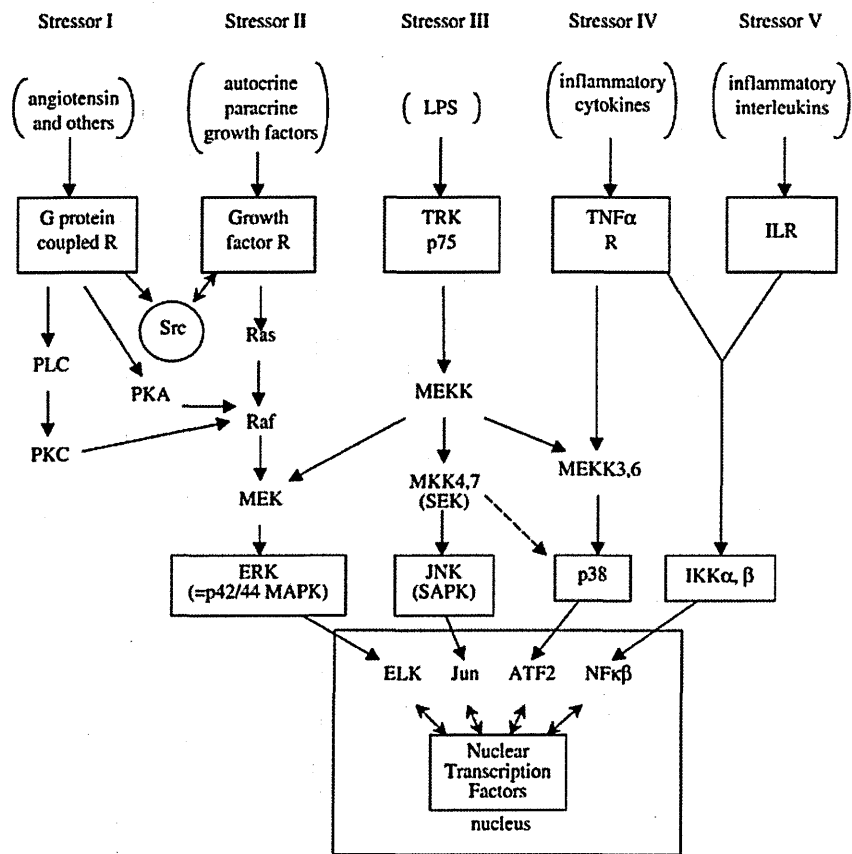
TCDD-induced changes in gene expression may also occur indirectly as a consequence of an AHR/ARNT-dependent induction of oxidative stress and the transcriptional activation of a further set of genes via a different enhancer, the so-called antioxidant response element (ARE) [43].

One of the major causes of dioxin’s toxicity is dioxin-evoked stress responses. It has been noted that dioxin causes typical cellular stress symptoms in affected cells. In

addition to oxidative stress, there are many documented signs of other types of cellular stresses induced by dioxin in various cell types.

It is well acknowledged that many forms of well-studied cell stressors utilize defined major pathways sometimes designated as stress-activated kinase pathways. Each stress-activated kinase pathway may be distinguished from others by a unique MAPK (here used as a generic designation) that not only mediates stress signal transduction, but also acts as the anchor component carrying the final message to the nucleus of the affected cell [44] (**figure 3**).

It has been suggested that one of the most active pathways in dioxin signalling is the extracellular signal-regulated protein kinases (ERK) pathway, whose dioxin-induced activation is mediated by the stress-induced growth factor TGF $\alpha$ , an autocrine factor produced and secreted by many types of cells into the extracellular matrix [45].



**Figure 3.** Schematic representation of major stress-activated kinase pathways. The pathway components and their interactions are very much simplified to illustrate the flow of major stress signalling.

#### ***1.1.4.2. Other mechanisms of action***

Although the evidence supports the hypothesis that most of the effects of TCDD are mediated through the AHR, there are several effects of TCDD that have been attributed to an AHR-independent mechanism.

For example TCDD has been reported to activate MAP kinases, such as Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), independently of AHR [46]. However, it remains to be clearly determined whether there is a cross-talk with AHR through the common regulator proteins shared by these different pathways.

Thus the mechanisms by which TCDD may interfere with signalling pathways appear to be extremely complex, and the elucidation of the resulting alterations in gene expression and their importance for the various aspects of TCDD toxicity clearly remains a continuing challenge.

#### ***1.1.5. TCDD as endocrine disruptor***

It has been widely reported that endocrine disruptors can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (*e.g.*, hormone synthesis, transport, and metabolism) have been shown to be equally important [31, 47].

Research on endocrine disruption effects and mechanisms is rather complex, since exposure to the same level of an endocrine signal during different life ages may produce different effects.

The prenatal and perinatal periods are particularly sensitive to dioxin exposure, and indeed, higher exposure doses are required to produce similar effects in adult animals.

Usually exposure in adulthood may be compensated by normal homeostatic mechanisms and may therefore not result in any significant or detectable effects. Moreover, because of cross talk between different components of the endocrine systems, effects may occur unpredictably in endocrine target tissues other than the system predicted to be affected [48].

Thus, for most reported associations between endocrine disruptors exposure and different biologic outcomes, the mechanism(s) of action are poorly understood and involve several different pathways.

Exposure to dioxins has the potential to disrupt multiple endocrine pathways and induce toxic responses. Experimental animal data have shown adverse effects in testicular function, including reduced sperm counts and motility [49-51].

Reproductive organs and the endocrine system are sensitive to TCDD during development and sexual maturation [52-54]. Compared to placental transfer, exposure via lactation plays a major role in offspring receiving maternal dioxins [55]. In *utero* and lactational TCDD exposure in rats has been associated with reduced ovarian weight and decreased estradiol [56]. Similar outcomes have been reported in primates, including decreases in estradiol and progesterone [57, 58].

Some evidences suggest that TCDD could alter human ovarian function, including steroidogenesis and ovulation [59, 60].

No definitive data are available for humans, but recently Mocarelli et al [10] observed that exposure to TCDD from infancy through puberty produces a reduction in serum 17beta-estradiol and a permanent effect of semen quality in human males as a result of the disruptive action of low concentrations of TCDD on the endocrine system.

It has been proposed that endocrine disrupters also affect non-reproductive tissues such as bone. Therefore, the next section considers bone biology and its regulation.



## **1.2. General aspects on bone**

### ***1.2.1. Bone tissue macroscopic organization: anatomy and physiology***

The skeletal system consists of cartilage and bone, a highly specialized connective tissue characterized from an organic mineralized extracellular matrix. Although macroscopically the skeleton seems to be a static organ, bone is an extremely dynamic tissue at the microscopic level.

In fact bone undergoes constant remodelling, which renews the tissue and maintains the bone mass constant [61].

Bone serves three main functions: mechanical, protective and metabolic. In fact the skeleton is the site of muscle attachment for locomotion and provides internal support to the body in all higher vertebrates protecting vital organs and bone marrow.

In addition to its supportive function, it has several metabolic functions and plays an essential role in maintaining blood calcium and phosphate levels.

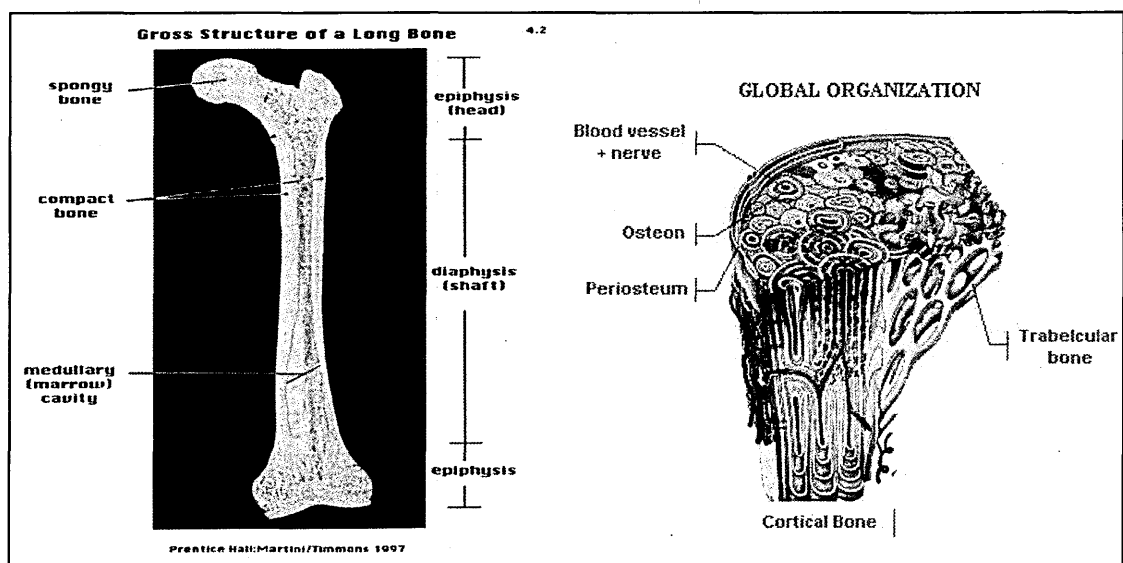
Morphologically, bone tissue is divided into cortical/compact bone, which forms about 80% of the mature skeleton, and cancellous/trabecular bone.

Both of them have the same matrix composition, but they differ structurally and functionally.

Cortical bone has higher density and less porosity compared to cancellous bone so the cortical bone fulfills mainly (but not exclusively) a mechanical and protective function and the trabecular bone a metabolic function.

Mature cortical bone consists of densely packed sheets of collagen lamella, concentric, parallel and interstitial, organized in a functional unit named an osteon or the Haversian System; whereas in cancellous bone the matrix is a meshwork of bars and spicules of bone lamella, thus it is more loosely organized.

Anatomically, bones are classified into three primary groups based on their general shape: short, flat, or long bones. Short bones (carpals and vertebral body, *e.g.*) are approximately the same in measurement in all directions and possess a trapezoidal, cuboidal, cuneiform, or irregular shape. Flat bones have one dimension that differs in length from the other two. The bones of the skull, scapula, mandible, and ilium are examples of flat bones. Long bones, such as tibia, femur, and humerus, consist of three anatomical divisions: the epiphyses, the metaphyses, and the diaphysis (**figure 4**). The diaphysis, or shaft of the bone, is a hollow cylinder in shape, surrounded by a layer of cortical bone. Diaphysis encloses the medullary cavity where the hematopoietic bone marrow is housed. The epiphysis is the ends of the bone and consists of a thin cortical layer of bone surrounding trabecular bone. The spaces enclosed by these thin trabeculae are also filled with hematopoietic bone marrow and are in continuity with the medullary cavity of the diaphysis. The metaphysis is the region of the bone between the epiphysis and diaphysis, containing both trabecular and cortical bone.



**Figure 4.** Schematic view of a long bone and its organization.

A plate of cartilage separates the epiphysis from the metaphysis and is known as the epiphyseal growth plate; this plate is the site at which elongation of long bones can occur. There are consequently two bone surfaces at which the bone is in contact with the soft tissues; both of them are lined with osteogenic cells organized in layers. The outer surface of the bone is known as the periosteum and the inner surface is known as the endosteum. There are two types of processes involved in bone development: intramembranous ossification (flat bones and bone formation occurring during bone remodelling) and endochondral ossification (long bones), characterized by the presence of a cartilaginous phase.

### ***1.2.2. Bone cells and bone matrix composition***

Four different types of cells reside in bone: 1) bone-forming osteoblasts, 2) osteocytes that sense mechanical strains and are trapped in bone matrix, 3) lining cells covering the bone tissue, 4) bone-resorbing osteoclasts.

#### ***1.2.2.1. Osteoblasts***

The osteoblast is the cell responsible for the production of the matrix constituents. It originates from local mesenchymal stem cells under the influence of several growth factors that will be described more in detail in *paragraph 1.3*.

Morphologically, active osteoblasts are cuboidal cells with a large nucleus, usually found in a single layer adherent to periosteal or endosteal surfaces of bone.

Since the main function of osteoblasts is the secretion of a complex mixture of bone matrix proteins (known as osteoid) active osteoblasts have a prominent Golgi complex and abundant rough endoplasmic reticulum. As polarised cells, they secrete osteoid

unidirectionally towards the bone surface on which they lie. Osteoblasts form tight junctions with adjacent osteoblasts, and possess specialized regions of their plasma membrane modified for vesicular trafficking and secretion. Osteoblasts are normally separated from the mineralized bone matrix by a thin layer of universalized matrix, the osteoid seam [62].

Osteoblasts also assist in the homeostasis of calcium by possessing receptors for the two primary calcium and bone regulating hormones, parathyroidhormone and 1,25-dihydroxyvitamin D (details in *section 1.2.4*).

Active osteoblasts may follow one of three courses once bone has been formed: 1) enclose themselves in bone, forming osteocytes, 2) remain on the bone surface, forming bone-lining cells, or 3) disappear from the site of bone formation by undergoing apoptosis.

#### *1.2.2.2. Osteocytes*

Osteocytes, the most abundant cells in bone, permeate the mineralized bone matrix occupying individual lacunae. In fact they derive from osteoblasts which become enclosed within the bone matrix during bone formation. Osteocytes have a typical morphology with long thin cytoplasmic processes, which form, in bone matrix, a fine network of connections (canaculi) with other osteocytes and with the osteoblasts and bone lining cells located at the surface of the bone [63]. They are believed to maintain the bone by responding to the mechanical stimuli and sensing bone damage.

#### *1.2.2.3. Bone lining cells*

Bone lining cells are mainly derived from osteoblasts that have become inactive. They are flattened, elongated cells covering quiescent the majority of the adult bone surface and thereby separate the bone surface from the bone marrow.

Bone lining cells are characterized from cytoplasmic extensions penetrating the bone matrix in order to get in contact with the extensions of osteocytes.

Bone lining cells are believed to be central in the maintenance of blood calcium and phosphate levels, and may have a role in attracting and stimulating osteoclasts to resorb bone.

#### *1.2.2.4. Osteoclasts*

Osteoclasts, responsible for the resorption of bone matrix during bone remodelling, are multinucleated giant cells arising from the fusion of mononuclear precursors, originating from hematopoietic stem cells in marrow. They contain from 1 to more than 50 nuclei and range in diameter from 20 to over 100  $\mu\text{m}$ .

In order to attend their bone resorbing role, they locate on bone surfaces tightly associated with the calcified matrix. Osteoclasts are polarised cells, having a ruffled border region of the cell membrane that is surrounded by an organelle-free region, or 'clear zone', which adheres to the bone surface via integrins, which are specialised cell surface receptors [63-65]. Osteoclastic bone resorption initially involves mineral dissolution, followed by degradation of the organic phase. These processes take place beneath the ruffled border and depend on lysosomal enzyme secretion and an acid microenvironment. Generally osteoclasts undergo apoptosis after a cycle of resorption, a process favored by estrogens. Bone resorption is strictly correlated with the maintenance of calcium homeostasis in the organisms, as described in *paragraph*

#### *1.2.3.*

#### *1.2.2.5. Bone matrix composition and organization*

Bone matrix makes up more than 90% of the volume of the bone tissue. The extracellular matrix contains 35% organic, 65% inorganic components and the water component is 10%.

Approximately 90% of the organic matrix consists mainly of collagen type I, which is organized in fibers preferentially oriented in a specific direction. The non-collagenous proteins are composed of non-collagenous glycoproteins and bone specific proteoglycans, including osteocalcin, osteonectin, bone phosphoproteins, bone sialoproteins and small proteoglycans. There are also a large number of proteins that are absorbed from the circulation. The non collagenous proteins have different functions in the regulation of bone mineralization, e.g. they mediate cell-to-matrix binding and act as growth factors.

The inorganic phase of bone matrix performs two essential functions: it is an ion reservoir and gives the bone tissue its stiffness and strength. Approximately 99% of body calcium, 85% of the phosphorous and 40-60% of total body sodium and magnesium are associated in mineral crystals in bone tissue. The physiologic concentrations of these ions in the extracellular fluid are thereby sustained. By forming hydroxyapatite-crystals ( $\text{Ca}_{10}[\text{PO}_4][\text{OH}_2]$ ) of calcium and phosphate the bone tissue is provided with stiffness and strength. Crystals tend to be oriented in the same direction as the collagen fibers. The preferential orientation of the collagen fibers alternates in adult bone from layer to layer, giving to this bone a typical lamellar structure.

The lamellae can be parallel to each other if deposited along a flat surface (trabecular bone and periosteum), or concentric if deposited on a surface surrounding a channel, the Haversian canal) containing blood and, sometimes, nerves. The Haversian system or osteon consists of such central canal, surrounding osteocytes and canaculi. The Haversian canals have an impressive network with transversely oriented canals,

Volkman's canals. The network of canals connects the periosteal and endosteal surface and the bone marrow enabling regulation of cell and bone metabolism.

However, when bone is being formed very rapidly (*e.g.* during development and fracture healing and some metabolic bone diseases), there is no preferential organization of the collagen fibers. They are not as tightly packed and found in somewhat randomly oriented bundles; this type of bone is called woven bone and is generally replaced in a second time by cortical tissue.

### ***1.2.3. Bone remodelling***

To carry out its metabolic function and the maintenance of the bone mass, bone is continuously resorbed by osteoclasts and subsequently rebuilt by osteoblasts that restore bone by synthesizing new bone matrix. This process is called bone remodelling, whose main function is to renew bone tissue to retain bone strength during adulthood [66].

Remodelling is most vigorous during the years of active growth, when deposition predominates over resorption.

Bone development and later remodelling are regulated by complex control systems that involve several types of intercellular signalling, between the osteoprogenitor cells and mature osteoblasts, osteocytes and osteoclasts.

Bone remodelling takes place in bone remodelling units comprising osteoblasts, osteoclasts and their precursors, in which resorption and formation are coupled [67].

Osteocytes are also marginally involved in the process by soluble factors or directly through cell to cell contacts. The bone remodelling cycle involves a complex series of sequential steps that are highly regulated. The main phases are 1) osteoclastic

resorption, 2) reversal phase, 3) preosteoblastic migration and differentiation into osteoblasts 4) osteoblastic matrix formation and 5) mineralization.

Bone formation and bone resorption do not, however, occur along the bone surface at random; they are either part of the process of bone development and growth or part of the turnover mechanism by which old bone is replaced by new bone. In the normal adult skeleton (*i.e.*, after the period of development and growth), bone formation occurs for the most part only where bone resorption has previously occurred. During the intermediate phase between resorption and formation (the reversal phase), some macrophage-like, uncharacterized mononuclear cells are observed at the site of the remodelling, and a cement line is formed, which marks the limit of resorption and acts to cement together the old and the new bone.

During skeletal development and bone remodelling throughout life, the cells from the osteoblast lineage synthesize and secrete molecules that in turn initiate and control osteoclast differentiation. Osteoblasts secrete two proteins, the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG), which are responsible for the communication between osteoclasts and their precursors (**figure 5**) [68, 69].

These two molecules have antagonistic effects on bone mass; while RANKL induces bone resorption, OPG blocks it. The receptor RANK is in fact required in conjunction with macrophage colony stimulating factor (M-CSF) for osteoclast differentiation.

OPG inhibits spontaneous or induced bone resorption. OPG acts as a decoy receptor that binds to RANKL and prevents it from interacting with its receptor [70].

RANKL and OPG, both of which are produced by osteoblasts at different stages of maturity, account for some of the signals in osteoblast–osteoclast communication.

The signal(s) that couples resorption and formation remains elusive, although several of the anabolic ligands, such as bone morphogenic proteins (BMPs) and transforming growth factor  $\beta$  (TGF $\beta$ ) are stored in bone matrix as bone is formed and are released

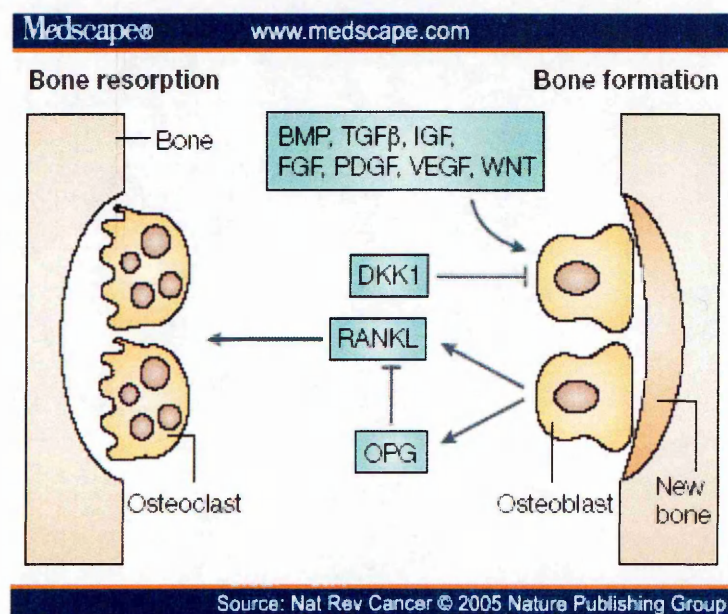


during bone resorption and can thus act on osteoblasts and precursors in the vicinity. BMPs could also enhance osteoclast differentiation from progenitors.

Besides that osteoclast activity is coupled to osteoblast activity, the activity of osteoblasts is also influenced by the activity of osteoclasts. During osteoclastic bone resorption multiple factors are released from the extracellular matrix, including insulin-growth factor I (IGF-I) and TGF $\beta$ , which control the differentiation and activity of osteoblasts after their release (*paragraph 1.3*).

Although bone resorption and formation are coupled, this process is not always in balance.

During growth, the balance favors bone formation to reach peak bone mass. After adults reach their peak bone mass there is evidence of a reduction of bone formation, which eventually results in bone loss and structural bone damage during ageing. The reduced bone mass, for instance, in osteoporosis results from an imbalance between bone resorption and formation, with the rate of resorption exceeding that of formation. In case of hormonal imbalance the coupling between osteoblasts and osteoclasts can also be out of equilibrium (*details in paragraph 1.2.4*).



**Figure 5.** Schematic representation of the communication between osteoblasts and osteoclasts during bone remodelling.

Many cytokines are also important factors for the normal bone remodelling sequence.

IL-1 is released by osteoblasts and it is a potent stimulator of osteoclasts. It works at all phases in the formation and activation of osteoclasts. Its effects are mediated through RANKL [71]. IL-6 is a pleiotropic cytokine expressed and secreted by normal bone in response to osteotropic hormones such as parathyroid hormone and dihydroxyvitamin D (*paragraph 1.2.4*). It has been implicated in the bone loss-associated skeletal metastases and with estrogen withdrawal in mouse models [72].

IL-15 and IL-17, both produced by T-cells, stimulate osteoclastogenesis and bone resorption [73, 74]. On the contrary, IL-18, a pro-inflammatory cytokine homologous to IL-1, inhibits osteoclast formation [75].

There are a number of *other factors* whose role in physiological and pathological bone remodelling is still to be clearly delineated. These include for example:

- *Retinoids*: Vitamin A excess eventually leads to increased bone resorption *in vivo* and hypercalcemia [76].
- *TGF-alpha*: TGF-alpha is a powerful stimulator of osteoclastic bone resorption. It stimulates the proliferation of osteoclast progenitors and probably acts on nonmature multinucleated cells. Its effects on bone cells are mediated through the epidermal growth factor (EGF) receptor [77].

#### ***1.2.4. Bone remodelling and systemic hormones***

The fact that bone remodelling occurs simultaneously in multiple skeletal locations is generally viewed as evidence that it is controlled locally, through autocrine and /or paracrine mechanisms [62]. This observation is also consistent with the possibility that bone remodelling is under endocrine control. Accordingly, several hormones are

already known to control bone remodelling. For instance, sexual steroid hormones such as estradiol are involved in the control of bone remodelling by affecting osteoclast differentiation and thereby bone resorption. As a result, the decline in levels of sexual steroid hormones at menopause is a predisposing factor for osteoporosis [62]. Like estrogens, androgens influence bone development with dramatic clinical manifestations. Decreased androgen levels have been linked to lower bone density in men, and there is a strong correlation between hypogonadism in elderly men and hip fracture and spinal osteoporosis [78].

Parathyroid hormone (PTH) has complex and only partially understood actions on bone. Paradoxically PTH has a catabolic effect (increase bone resorption) and an anabolic effect on bone (increase bone formation) depending on the dose and on the mode of administration (continuous versus intermittent) [79].

Another systemic hormone such as dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}_3$ ] has a multiple and complex effects on bone. Like PTH, it stimulates osteoclastic bone resorption by stimulating differentiation of osteoclast progenitors but also activates mature osteoclasts.

On the contrary, calcitonin is potent inhibitor of bone resorption, but its effects are only transient.

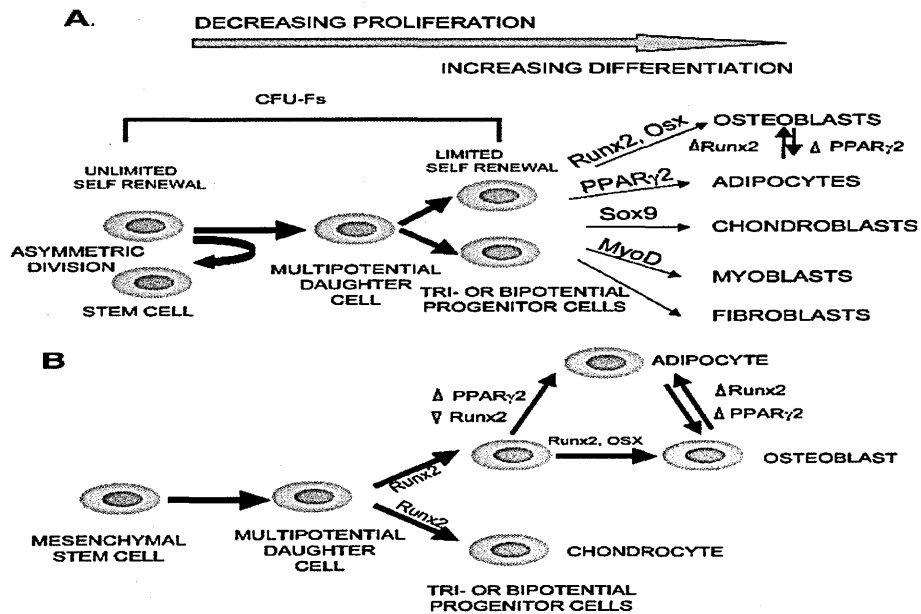
Other systemic hormones are important in regulating skeletal growth. The growth hormone is "anabolic", which means it stimulates bone formation, glucocorticoids are necessary for bone cell differentiation during development and thyroid hormones can also stimulate bone resorption and formation and are critical for maintenance of normal bone remodelling.

### 1.3. Osteoblast lineage

The osteoblastic lineage cells that mediate bone formation are comprising the following phenotypes: *mesenchymal stem cells* that give rise to osteoprogenitor cells as well as the cells of other lineages; *osteoprogenitor cells* that contribute to maintaining the osteoblast population and bone mass; *pre-osteoblasts*, cells that started the differentiation process but not yet synthesising bone matrix; osteoblasts that synthesise the bone matrix on bone forming surfaces [80]; *osteocytes*, organised through the mineralized bone matrix that support bone structure, and *lining cells* that protect the bone surface.

#### 1.3.1. Mesenchymal stem cells

Postnatal bone marrow stroma contains cells that have both significant proliferative capacity and the capacity to form osteoblasts, chondroblasts, adipocytes and myoblasts under appropriate conditions [81-83]. These are mesenchymal-derived stem cells, commonly referred to as mesenchymal stem cells (MSC) or stromal cells and are distinguished from the hematopoietic stem cell lineage present in bone marrow. Commitment of MSCs to tissue-specific cell types is orchestrated by transcription factor regulators that serve as "master switches". As shown in the **figure 6**, two transcription factors, Runx2 and Osterix (Osx) are absolutely required for osteoblast differentiation [84, 85].



**Figure 6.** Stem cell commitment to mesenchymal phenotypes. A schematic of stem cell commitment through either (A) a single step process or (B) a multistep hierarchical process with increasing lineage restriction to various end-stage mesenchymal cell types. Some of the known transcription factors playing key regulatory roles in the mesenchymal lineages are indicated. Also depicted is apparent plasticity between osteoblasts and adipocytes. [86].

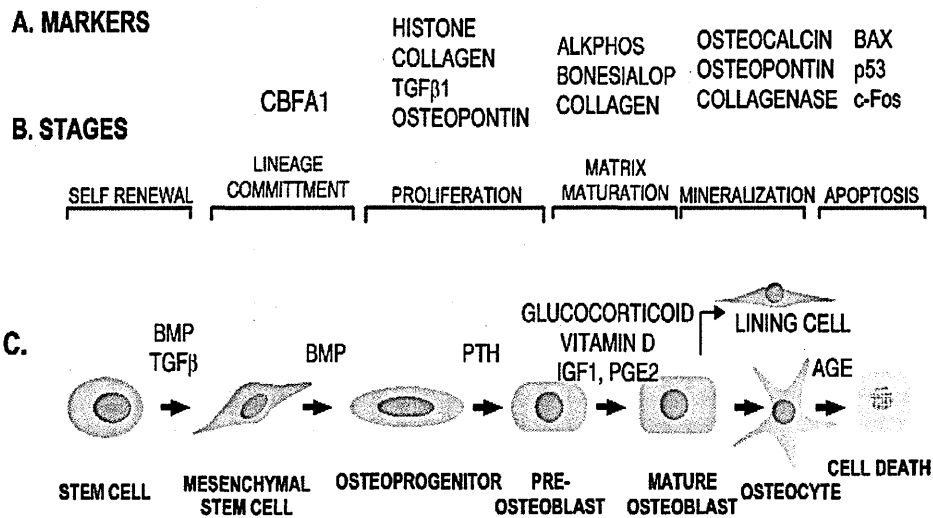
### 1.3.2. Osteoblast differentiation process

The progression of osteoblast maturation requires a sequential activation and suppression of genes that encode the phenotypic and functional proteins of the osteoblast populations. Signalling proteins, transcriptions factors and regulatory proteins support the temporal expression of genes that characterize stages of osteoblast differentiation and bone formation.

As shown in **figure 7**, the process of osteoblast differentiation can be divided in three main subsequent steps: (i) proliferation, (ii) matrix synthesis and maturation and (iii) mineralization. Each stage is characterized by the expression of distinctive osteoblast markers. Most frequently used markers of osteoblastogenesis are: transcription factor RUNX2 (called also cbfa1) which is expressed in a period of active proliferation,

alkaline phosphatase (ALP) in matrix maturation, and osteocalcin (OCN) in the onset of mineralization [87, 88].

The ordered temporal expressions of these genes were used as specific markers for osteoblastic differentiation in this study.



**Figure 7.** Growth and differentiation of osteoblasts. Schematic illustration of (A) frequently used markers of (B) osteoblast maturation stages. (C) The bone morphogenetic proteins (BMP2/4/7) are required for lineage direction. At each stage of maturation, cells are responsive to steroid and polypeptide hormones that promote their further differentiation, as well as mediating their functional activities (*e.g.*, PGE2, IGF-1). Selected examples of regulatory factors are shown [86].

*1.3.2.1. Transcription factors controlling osteoblastogenesis*

The role of the most important transcription factors governing osteoblasts differentiation is summarized here.

*Runx2*

Runt-related transcription factor 2 (Runx2, called also cbfa1) is the earliest marker of osteoblast lineage cells and it is necessary, but not sufficient, for a progenitor cell to differentiate along the osteoblast lineage.

Runx2 operates in bone lineage cells mainly by binding to the Runx consensus sequence 5'- PYGPYGGT-3', named also osteoblast specific element (OSE2).

The Runx regulatory element can be found in the promoter of all major osteoblast genes controlling their expression, including type I collagen alpha 1 chain, osteopontin, vascular endothelial growth factor (VEGF), RANKL, sclerostin, bone sialoprotein and osteocalcin, resulting in the establishment of an osteoblast phenotype. Additionally, Runx2 can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway by binding of type I collagen to alpha2beta1-integrins on the osteoblast surface [89].

In addition to control osteoblast differentiation, Runx2 was found to negatively control osteoblast proliferation by acting on the cell cycle [90]. Moreover Runx2 can modulate the expression of kinases such as p85 PI3K that controls osteoblast differentiation and survival [91]. Recent studies indicate that Runx2 interacts with several regulatory proteins within the nuclear architecture, resulting in activation or repression of genes which control the program of osteoblast proliferation and differentiation [92]. This indicates that Runx2 can control osteoblastogenesis through multiple mechanisms.

### *Osterix*

Osterix (Osx), also known as SP7 transcription factor, is zinc finger transcription factor essential for the formation of preosteoblasts from multipotent progenitors. In fact Osx-deficient mice show absence of osteoblasts and defective bone formation [93]. However, Runx2 is expressed in Osx-deficient mice, indicating that Osx acts downstream of Runx2. Osx transcription is positively governed by Runx2 and acts by directing pre-osteoblasts to immature osteoblasts; Osx in turn, impairs osteoblast differentiation. Although little is known on the mechanisms of action of Osx, this protein was found to form a complex with the nuclear factor of activated T cells (NFAT), resulting in activation of type I collagen alpha promoter activity [94].

Accordingly, constitutive activation of NFAT activates the Wnt signalling pathway, bone formation and bone mass. Osterix is also a suppressor of VEGF expression following endothelin-1 exposure; therefore, it might be a crucial mediator of osteoblast function within the context of bone remodelling.

### *β-Catenin*

Genetic studies have revealed that canonical Wnt signalling is an important pathway controlling bone formation and bone mass [95, 96]. Inactivation of β-Catenin blunts osteoblast differentiation from mesenchymal progenitors, indicating that β-Catenin plays an essential role in osteoblast differentiation *in vivo* [97]. One of the targets of Wnt signalling is Runx2 as β-Catenin/TCF1 promotes Runx2 expression and activity [98]. One Wnt protein, Wnt10b, was found to induce the expression of Runx2, Osx and Dlx5 and to reduce the expression of adipogenic transcription factors C/EBPα and PPARγ in mice, indicating that Wnt signalling controls osteoblastogenesis and bone mass by modulating several transcription factors [99].

### *ATF4*

ATF4 or CREB2 (cAMP response elements binding protein2) is another important transcription factor controlling osteoblasts. ATF4 interacts with Runx2 to regulate the transcriptional activity of osteocalcin [100]. Additionally, ATF4 can be phosphorylated by the kinase Rsk2 and thereby controls amino acid transportation in osteoblasts, an important step in bone formation. ATF4 is a ubiquitous protein expressed in many cells but it is degraded in osteoblasts after its ubiquitination. ATF4 is therefore a transcription factor whose activity is regulated at the post-transcriptional level [101]



### AP1

AP1, a transcription factor composed of heterodimeric proteins of the Fos and Jun families (c-Jun, JunB et JunD; *c-Fos*, FosB, Fra-1, Fra-2) is an important regulator of bone formation, as demonstrated by genetic manipulations in mice [102]. *c-Fos* is the first member of the AP1 family of transcription factors that was recognised to regulate osteoblast proliferation and gene expression [103]. More recent studies showed that other members control osteoblast differentiation. *In vivo*, overexpression of Fra-1 or  $\Delta$ FosB (a splice variant of FosB) promotes osteoblast differentiation and thereby bone formation and bone mass resulting from increased number of osteoblasts [104]. On the contrary, down-regulation of Fra1 or JunB results in reduced bone formation and bone mass in mice [105]. Besides direct effects on osteoblast gene transactivation, some AP1 members can act with other transcription factors. For example, Jun proteins can heterodimerize with ATF4 to control gene expression [106]. Thus, the AP1 family of transcription factors can control osteoblast differentiation by acting at multiple levels.

### PPAR $\gamma$ 2

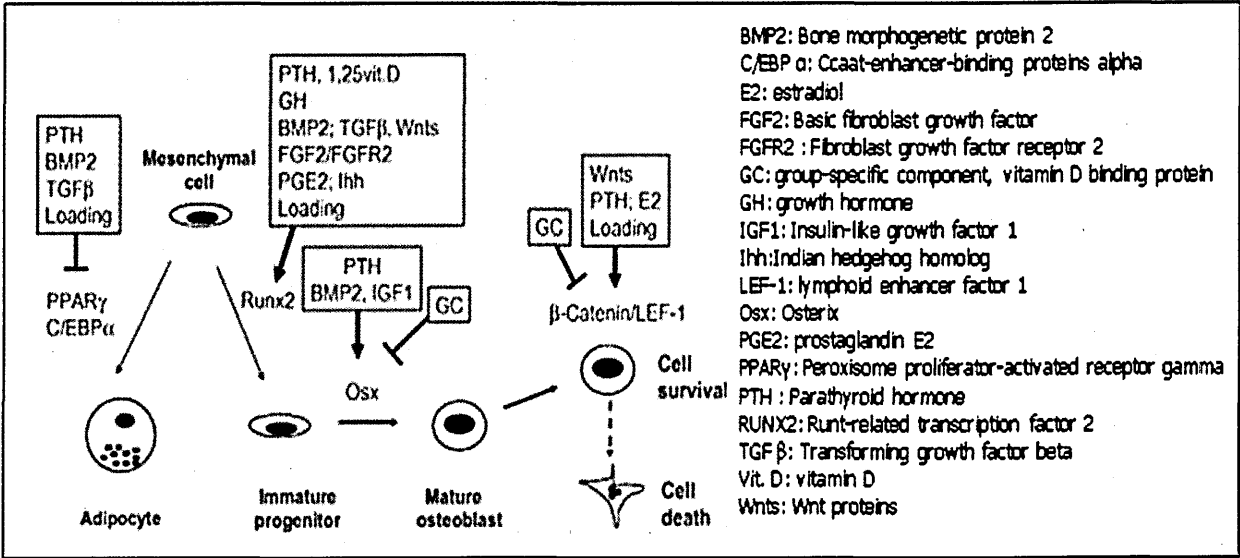
In addition to Runx2, other transcription factors play a role in lineage determination. Proliferation-activated receptor  $\gamma$ 2 (PPAR $\gamma$ 2) is known to be involved in the differentiation of mesenchymal cells into osteoblasts. Consistently, increased expression of PPAR $\gamma$ 2 induces adipocyte differentiation and reduces osteoblast differentiation in mesenchymal cells [107] whereas, as expected, forced expression of Runx2 in adipocytes results in osteoblast differentiation [108]. In addition to inducing adipocyte gene expression, PPAR $\gamma$ 2 can bind Runx2 and inhibit its transcriptional activity. Consistent with these *in vitro* data, PPAR $\gamma$ 2 removal in mice promotes osteoblast differentiation resulting in increased bone mass [109]. The role of these transcription factors in the inverse relationship between osteoblastogenesis and

adipogenesis in the bone marrow stroma in osteopenic disorders and aging remains, however, to be determined.

1.3.2.2. Regulation of transcription factors in osteoblasts

Numerous secreted factors of paracrine, autocrine, and endocrine origin influence osteoblast development and maturation.

**Figure 8** summarizes the effects of many local and external factors which exert positive or negative effects on osteoblast commitment, differentiation and survival.



**Figure 8.** Regulation of transcription factors during osteoblastogenesis [85].

Many *hormones* such as PTH, estrogens, glucocorticoids and vitamin D control bone formation by regulating the expression of transcription factors in osteoblasts. For example PTH promotes bone formation through phosphorylation and activation of Runx2, resulting in activation of osteoblast genes [110]. Estrogens favour osteoblastogenesis by activating Runx2 and /or Wnt/beta-Catenin-mediated osteoblast survival [111]. The Wnt signalling pathway is only relatively recently known to be involved in bone metabolism. Wnt signalling plays a widespread role in skeletogenesis

from embryonic skeletal patterning, through fetal skeletal development, and bone remodelling in adults. Finally both growth hormone (GH) and vitamin D upregulates Runx2 expression, thus favoring osteoblastogenesis [112].

In addition to hormonal factors, most *growth factors* control osteoblastogenesis by modulating transcription factors expression and/or activity.

Notably, bone morphogenic protein BMP-2 promotes Runx2 expression in mesenchymal osteoprogenitors [113]. It also promotes Osx expression in osteoblastic cells.

Although transforming growth factor- $\beta$  (TGF $\beta$ ) was found to inhibit Runx2 activity *in vitro* TGF $\beta$  promotes bone formation *in vivo* by increasing Runx2 and decreasing PPAR $\gamma$ 2 expression [114]. Another anabolic factor, insulin-like growth factor-1 (IGF-1), promotes Osx in osteoblastic cells. The anabolic factor fibroblast growth factor-2 (FGF-2) increases Runx2 phosphorylation and activity. Consistently, activation of FGF receptor 2 (FGFR2) expression or signalling results in increased Runx2 expression and enhanced osteoblast differentiation [115]. The *in vivo* anabolic effects of PGE2 on bone formation are also associated with increased expression of Runx2 [116].

Finally, anabolic Wnt proteins, such as Wnt3a and Wnt10, upregulate Runx2 expression by activating the Wnt/ $\beta$ -Catenin pathway. Additionally, Wnt signalling stimulates osteoblastogenesis of mesenchymal precursors by suppressing C/EBP and PPAR $\gamma$  [117]. Thus, the action of these regulatory factors on osteoblastogenesis is likely to be mediated at least in part by modulation of transcription factors.

#### 1.4. Dioxin and bone

It has been suggested that endocrine disrupters could contribute etiologically not only to reproductive-related disorders but also to disorders involving non-reproductive tissues, such as brain and skeleton, whose safety is regulated by endocrine system.

Exposure to endocrine-disrupting chemicals, such as dioxins, may represent a risk factor for bone development and diseases, given that the incidence of osteoporosis is rising in the Western world [118], and experimental data shows that organochlorines have adverse effects on bone quality [119].

Bone development and homeostasis are controlled by a complex interaction of several hormones and vitamins [120], whose balance can be disturbed by low levels of dioxin, so bone may well be a target for the effect of TCDD.

Rather recently, it has been reported that *in vivo*, long-term exposure to TCDD dose dependently interferes with bone growth, modelling, and mechanical strength in adult rats [121].

Similarly, exposure of pregnant rats to a single dose of TCDD resulted in altered bone geometry, mineral density, and mechanical strength in the offspring [122]. In addition, the both studies suggested that the AHR plays a role in modulating the effects of dioxins on bones, because dioxin-resistant rats carrying a deviant aryl hydrocarbon receptor (AHR) were more resistant to TCDD-induced bone effects than wild-type rats. Interestingly, these bone effects were observed at doses of relevance for environmental health risk assessment [121, 122].

Furthermore, data from *in vitro* studies suggest that proliferation of osteoprogenitor cells and their differentiation into mature bone cells can be target of TCDD.

It has been shown that in osteoblasts isolated from neonatal rat calvaria, 1 nM TCDD significantly reduced alkaline phosphatase enzymatic activity and this reduction was shown to occur by an AHR-dependent mechanism [123]. Furthermore, data from *in*

*vitro* studies showed that TCDD suppresses differentiation of normal diploid rat osteoblasts [124]. Another AHR ligand, 3-methylcholanthrene (3MC), also inhibited differentiation and proliferation of rat and mouse osteoblastic cells [125]. These observations are in accordance with recent data indicating that TCDD exposure hampers matrix maturation [126].

Despite these studies, the detailed mechanisms of TCDD-induced bone effects are still unknown.

In the view of the current lack of information on these mechanisms, it would be appropriate to investigate this aspect further. Although several experimental approaches are possible the present study has used proteomics. It is therefore timely to consider this technology and how it might be applied to gain more information on possible mechanisms of TCDD-induced bone effects.

## 1.5. Proteomics

### ***1.5.1. Proteomics in the field of toxicology: toxicoproteomics***

Proteomics refers to the study of the proteome, which is the total protein complement of a genome. It is presently considered to be the key technology in the global analysis of protein expression and in the understanding of gene function and regulation in the postgenomic era [127].

Proteins are cell- and tissue-specific and are affected by age, disease and trauma. Thus proteomes are temporally and spatially dynamic, with each analytical profile representing a unique moment in time. The scope of proteomics is broad; it encompasses identification and quantitation of proteins in cells, tissues, and biological fluids; analysis of changes in protein expression in normal *versus* diseased cells or between cells grown under different conditions; characterization of post-translational modifications; and studies of protein-protein interactions [128-130].

Toxicoproteomics uses the discovery potential of proteomics in toxicology research by applying global protein measurements technologies to biological samples after exposure to injurious agents.

Expression analysis at the protein level is a powerful new tool in toxicology, which allows the simultaneous identification of multiple proteins that are up- or down-regulated in response to a toxic event, thus providing a global perspective about how an organism responds to a specific toxicant. This information can define cellular gene and protein networks, identify target molecules of toxins, and provide future biomarkers and novel testing procedures.

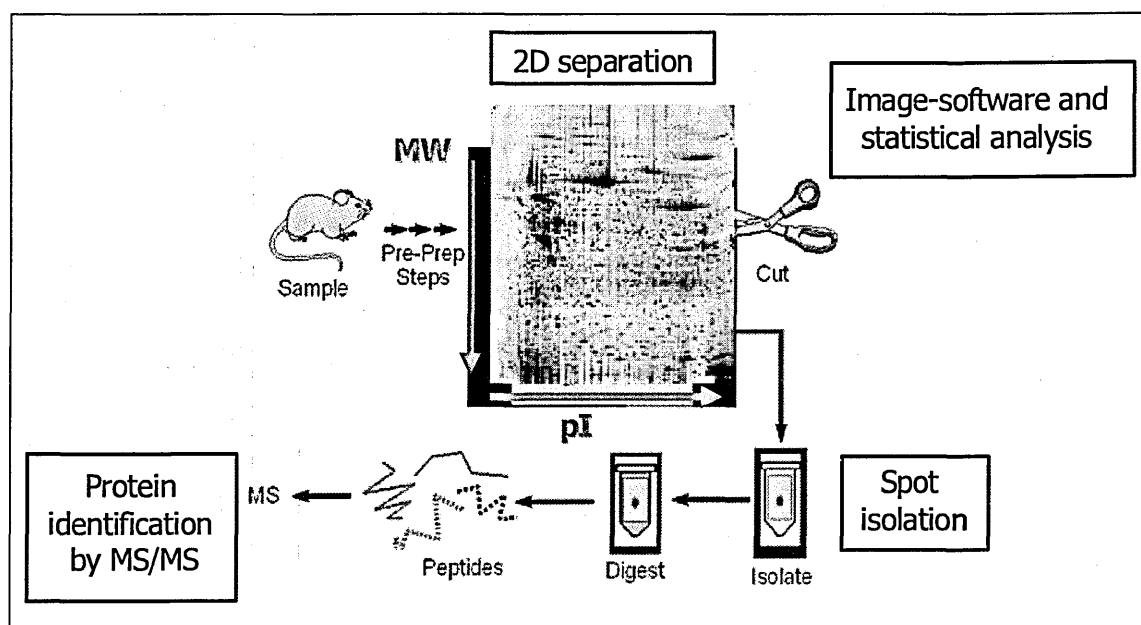
Toxicoproteomics seeks to discover critical proteins and pathways responding to adverse chemical exposures and environmental stressors, in order to provide new toxicity signatures. In toxicoproteomics, the full range of proteomics methods and

technologies are used to uncover the cellular and subcellular mechanisms at work in response to exposure to xenobiotics.

However, the use of toxicoproteomics in environmental sciences as a means for understanding toxic response and mechanisms is still relatively undeveloped, but has the potential to revolutionise our ability to characterise hazards [131-134].

### 1.5.2. Introduction to proteomic analysis

The proteomic workflow used in this study is shown in **figure 9**.



**Figure 9:** Proteomic workflow

It comprises four main steps: (1) sample preparation, (2) separation of the proteins by two dimensional gel electrophoresis (2-DE), (3) differential gel image analysis and statistical analysis, (4) in gel digestion and extraction of peptides (5) identification of the protein of interest by tandem mass spectrometry (MS/MS) analysis of these peptides.

Both 2-DE and mass spectrometry analysis have been very successfully employed, but need appropriate statistical analysis and sophisticated bioinformatics tools to minimize 2-DE experimental artefacts and avoid false positives or negatives in MS/MS protein identification [135-137].

#### *1.5.2.1. Sample preparation*

Appropriate sample preparation is absolutely essential for good 2-DE results. Due to the great diversity of protein sample types and origins, the optimal sample preparation procedure for any given sample must be determined empirically. An efficient and reproducible method is essential to get reliable analysis results.

Ideally, the process will result in the complete solubilization, disaggregation, denaturation, and reduction any disulphide bonds of the proteins in the sample. To fully analyze all intracellular proteins, the cells must be effectively disrupted. Choice of disruption method depends on whether the sample is derived from cell suspensions, solid tissue, or other biological material, and whether the analysis is targeting all proteins or just a particular sub-cellular fraction. Moreover the protein sample should be protected from proteolysis during cell disruption and subsequent preparation.

#### *1.5.2.2. Two-dimensional gel electrophoresis*

2-D electrophoresis, introduced by O'Farrell in 1975, is a powerful and widely used method for the analysis of complex protein mixtures extracted from different biological samples. This technique separates thousands of proteins simultaneously according to two independent properties in two discrete steps.

*The first-dimension* step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the ionisable groups present and



the pH of their environment. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl-termini. The isoelectric point is the pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below and negatively charged at pH values above their pI. Under the influence of an electric field, a protein will migrate to the position in a pH gradient where its net charge is zero. This is the focusing effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated based on very small charge differences. The development of immobilized pH gradients (IPG-strips) for the first dimension of 2-DE, pioneered by Gorg et al. [138] made 2-DE the most useful and suitable tool to separate thousands of proteins simultaneously.

*The second-dimension* step, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights ( $M_r$ , relative molecular mass). Proteins will thus migrate to a co-ordinate on a 2D gel defined by their molecular mass and pI. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the relative amount of each protein can be obtained.

Visualization of the proteins after their separation can be done with different staining techniques. Organic dyes like Coomassie R250 or Coomassie G250 (colloidal Coomassie) are widely used. Coomassie staining has a linear response of three orders of magnitude but it suffers from a low detection sensitivity in protein, though the colloidal version is somewhat better in this respect [139]. Despite this limitation, colloidal Coomassie is still widely used, because of its low price, ease of use and compatibility with most subsequent protein analysis and characterization methods. Other methods include silver staining, which is generally more sensitive, though individual proteins are quite variable in their response and the range of linearity is rather limited. Some silver staining protocols have poor compatibility with subsequent MS analysis [140]. Fluorescent dyes such as Sypro Ruby, have a sensibility that is

comparable to silver staining but their response is linear over a greater range of protein concentration (at least three orders of magnitude [141]). Being end point stains the protocol is very simple, and they are highly compatible with in gel digestion and mass spectrometric analysis [142] but they are unfortunately rather expensive.

#### *1.5.2.3. Computer assisted 2-DE image analysis*

The reliable evaluation and comparison of the complex 2-DE patterns by eye is problematic. Therefore the gel images have to be converted into digital data with a scanner and analysed by image analysis software. For an optimum computational analysis it is important to obtain a high resolution grey scale image ideally with a bit depth of 16 to maximise the number of distinguishable grey levels, so maintaining dynamic range.

The success of differential expression analysis depends critically on the accuracy and the reliability of the analytical software used for image processing. The software and the parameters chosen may have a profound effect on the interpretation of the protein pattern obtained and the amount of user intervention demanded during the analysis [143].

Currently, several 2-DE image analysis software packages are commercially available. These programs have been continuously improved and enhanced over the years in terms of faster matching algorithms with less manual intervention and with focus on automation. Novel algorithms offering more reliable spot detection, quantitation and matching have been introduced together with the inclusion of flexible statistic tools for a more robust differential expression analysis. This is the case of the state-of-the-art image software PG240 and Same Spots (NonLinear Dynamic, UK). These programs provide fully automated gel image processing (spot detection, gel alignment, spot matching and spot quantification) at the pixel level, along with sophisticated data

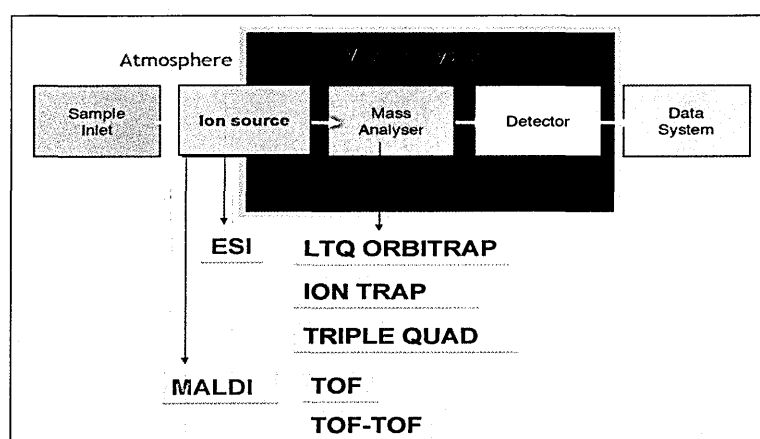
visualization and downstream analysis tools. More details on their characteristics and their use are reported on the Material and Methods section (*section 3.10*) [143-145].

## 1.6. Protein identification by Mass-Spectrometry

### 1.6.1. Principles of Mass Spectrometry

Mass Spectrometry is an analytical technique that measures the mass:charge ratio of a charged species based its response to electric and / or magnetic fields. The sample molecules are converted into positive or negatively charged gas phase ions and are separated according to their mass to charge ( $m/z$ ) ratios [146, 147].

As shown in **figure 10**, mass spectrometers consist of *three basic components*: an ion source, a mass analyser and a detector.



**Figure 10.** A schematic indicating the main regions of a mass spectrometer, and indication principle ionization methods and types of mass analyser.

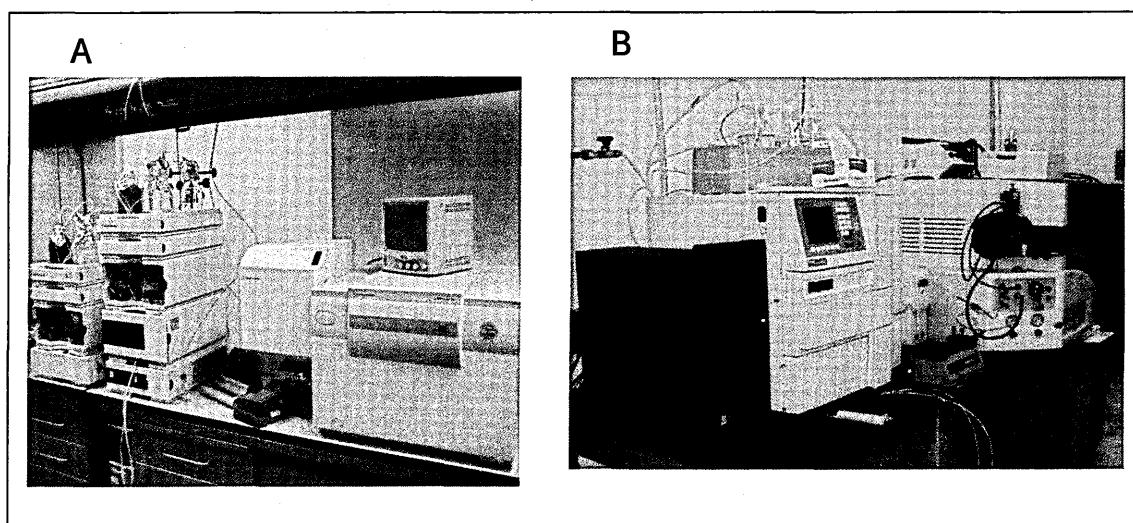
The ion source converts sample molecules into ions in gas phase and one or more mass analysers separate the various ionised species according to their  $m/z$  ratio, while the detector records signal intensity at each  $m/z$  value. Conventionally, mass spectra are presented as a plot of  $m/z$  value against relative intensity.

Currently, the two most common ionisation methods used in the analysis of peptides and proteins are matrix assisted laser desorption ionisation (MALDI) and electrospray ionization (ESI); the latter was almost exclusively used in my studies.

A number of different types of mass analysers are employed in proteomic research, and they are listed in **figure 10**.

Different types of mass spectrometers used in proteomics differ in their physical principle, their performance standards, their mode of operation and their ability to support specific analytical strategies [148-150].

In this thesis, two different mass spectrometers were utilised for protein identification, in order to take advantage from their complementary qualities: the first one used was the ESI-IT (ion trap) MS/MS (**figure 11 A**), ESI Q-TOF (quadrupole–time of flight) MS/MS (**figure 11 B**) whose components will be described more in detail below.



**Figure 11.** (A) An overview of our Agilent 1200 LC/MSD Trap XCT. (B) A representative image of the Waters Micromass Q-ToF micro Mass Spectrometer.

Mass spectrometry based proteomics fall into two different families, top down and bottom up [150, 151].

In the top-down approach, intact protein ions are introduced into the gas phase and their masses are measured, often at high accuracy with, for example, a Fourier transform spectrometer, providing the molecular mass of the protein. Subsequent fragmentation may provide additional structural information in the form of partial amino acid sequences.

In the more widely used bottom-up approach, proteins are digested with a proteolytic enzyme, and the resulting peptides are analyzed in the gas phase by mass spectrometry (MS) or by tandem mass spectrometry (MS/MS).

In MS analysis, the masses of the intact peptides are determined and protein identification relies upon a comparison of the experimentally determined MS peak mass values with the predicted molecular mass values of the peptides generated by a theoretical digestion of each protein in a database (peptide mass fingerprinting).

In MS/MS analysis, peptide ions are separated in a first mass analyser. Selected parent ions are fragmented and the fragment ions are separated in a second mass analyser to produce information on the sequence of the peptide. The resulting tandem mass spectra are compared with computer generated fragmentation models derived from all the tryptic peptides computed from the entries in a protein or translated nucleic acid database.

### ***1.6.2. Protein cleavage***

Most strategies for protein identification by mass spectrometry require proteins to be proteolytically cleaved into a mixture of shorter peptides which fall within the operating mass range of the spectrometer. This is especially the case for 2D gel based workflows, as used in the present studies, since in gel digestion provides a simple and efficient way of recovering the stained gel-entrained protein for subsequent characterization by MS.

The most widely used enzyme is trypsin, which hydrolyzes the protein on the C-terminal side of lysine and arginine, unless the following amino acid is proline. This is advantageous as every peptide other than the one including the original C terminus has at least two sites for efficient protonation, the N-terminal amino group and the C-

terminal basic residue, so peptides are readily ionized and detected as positive ions. Moreover the presence of a basic residue at the C terminus favours simple and easily interpretable fragmentation pathways in CID experiments (see below).

### ***1.6.3. Sample introduction into the mass spectrometer***

The method of sample introduction will depend of the complexity and purity of the sample, and the mode of ionization employed. Well desalted samples, containing relatively few components of reasonable abundance may be infused directly; in most cases however online liquid chromatography is the favoured introduction method, particularly in the case of ESI, which, as an atmospheric pressure, solution phase technique is intrinsically compatible with liquid chromatography.

The studies reported in this thesis used mainly reversed phase liquid chromatography (RP-LC). In this technique the stationary phase is non-polar with respect to the mobile phase and hydrophobic interactions are the main mechanism of analyte retention on the column. The RP-LC columns most commonly used in peptide separations are packed with a stationary phase in which non-polar C18-alkyl chains are covalently attached to silica particles.

Reversed phase chromatography usually achieves a considerable improvement in the concentration of samples entering the ionization source, since relatively large volumes of dilute sample in aqueous solution can be pumped through the column, with the analytes of interest being initially retained as a narrow band at the top of the column, and components being sequentially eluted as the proportion of organic solvent in the mobile phase is increased. This concentration effect is enhanced by columns of small bore as the same amount of sample will be eluted in a lower volume. The use of nano-

scale LC columns with internal diameters (i.d.s) of 75  $\mu\text{m}$  or less has dramatically improved the sensitivity of analysis of complex biological samples [152].

The signal to noise ratio is generally further improved because most ionic buffers and inorganic salts are not retained on RP columns, and so can be diverted to waste. Reversed phase chromatography generally increases the number of peptides detected in complex mixtures, because fewer separate components enter the source at any one time, so the duty cycle of the spectrometer is likely to be more efficiently exploited.

#### ***1.6.4. Ion sources***

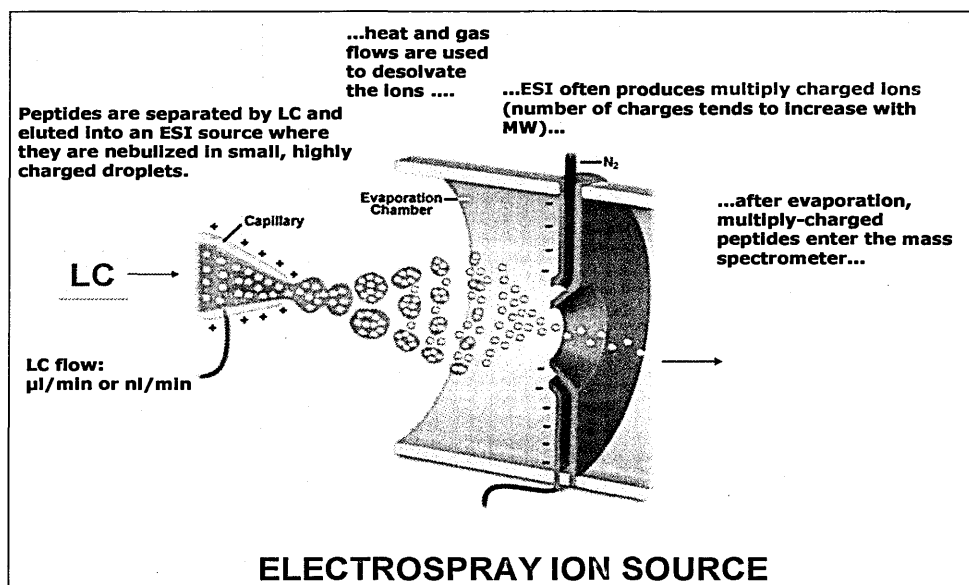
The spectrometers used in my studies were both equipped with electrospray ionisation sources.

ESI was first described by Dole and was developed as a practical ionization source for mass spectrometry, initially on quadrupole instruments by John Fenn [153]. The mechanism of electrospray is visualized in **figure 12**. It is an atmospheric pressure ionization technique in which sample solution is sprayed from a fine capillary (usually around 10 $\mu\text{m}$  in diameter) which is maintained at a high potential difference with respect to the ion source orifice of the spectrometer.

This voltage is applied either via direct contact with an electrode, or through a (metallic-) coating on the spray tip. The potential difference generates a force extending the liquid to form a cone at the capillary tip (the Taylor cone) from which a fine mist of electrically charged droplets emerges. Positive charge is conferred by the presence of protons, or other cations.

These droplets rapidly become smaller, due to evaporation of solvent molecules until Coulombic repulsion causes fission, forming smaller droplets with reduced charge density.





**Figure 12.** Schematic of ESI (from web sources).

There are two mechanisms proposed for the desolvation and charging of analytes within the droplets: ion evaporation and charge residue models (IEM and CRM, respectively).

The IEM was the first mechanism to be proposed. This model states that when the droplets become smaller, the electric field will become strong enough to expel ions into the ambient gas. Evaporated ions can remain somewhat solvated. This model applies mainly to small ions.

For the ionization and evaporation of proteins, *i.e.* considerably large ions, the CRM is more applicable. In this model large droplets dissociate via a sequence of Rayleigh instabilities, called Coulombic explosions, into successively smaller ones until eventually only one analyte molecule per droplet remains. Further evaporation of solvent results in fully desolvated charged protein molecule. The charges are statistically distributed over the analyte's potential charge bearing sites, enabling the formation of multiply charged ions.

### ***1.6.5. Mass analysers***

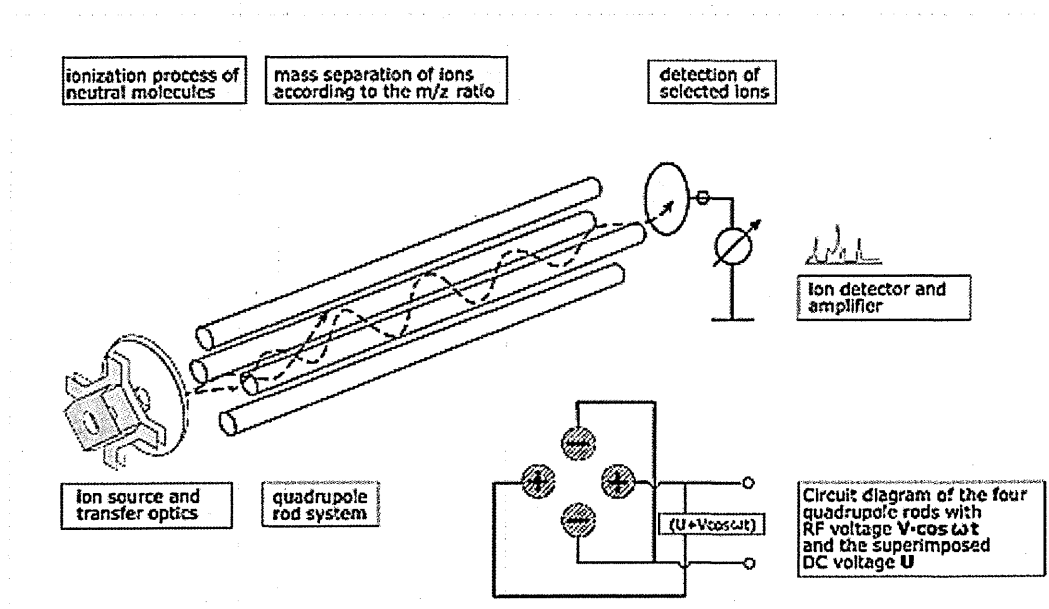
The mass spectra reported in this thesis were obtained using either a Micromass Q-ToF or an Agilent LC MSD or a spectrometer. These systems employ time of flight, quadrupole or quadrupole ion trap mass analysers.

#### ***1.6.5.1. Quadrupole mass analysers***

The quadrupole mass analyser separates ions according to their  $m/z$  ratio by utilising the stability of their trajectories in an oscillating electrical field. Ions that do not have a stable trajectory through the quadrupole are ejected from the device and do not reach the detector.

The electric fields are provided by applying DC and radiofrequency voltages to opposite pairs of parallel hyperbolic rods (**figure 13**).

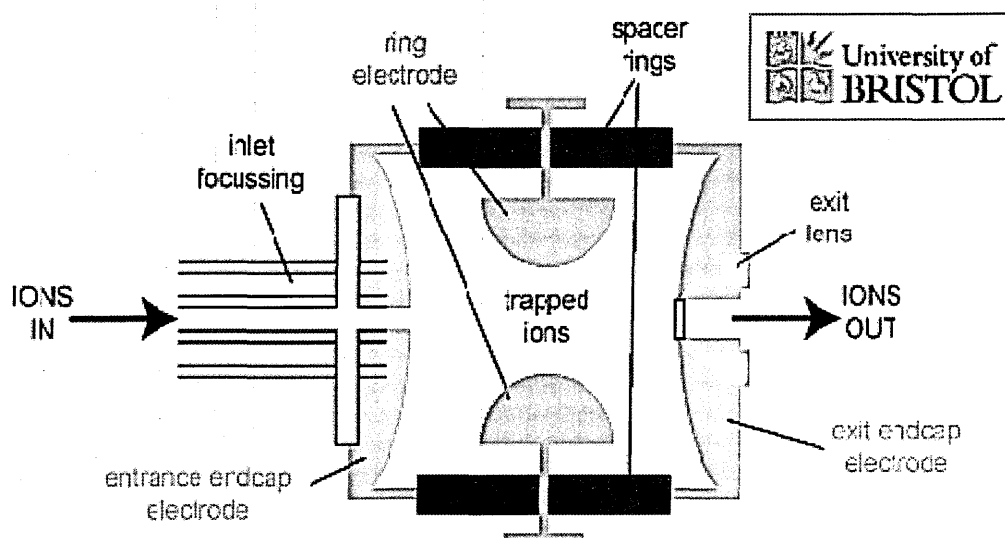
If the DC voltage is removed, and the quadrupole is operated in radiofrequency only mode, it functions as a wide band pass filter, and all ions are transmitted. Application of the DC voltage enables transmission of a selected mass, and scanning of the DC voltages allows sequential transmission of a range of masses, so providing a conventional mass spectrum.



**Figure 13.** Operating principle of a Quadrupole Mass Spectrometer (from web sources).

#### 1.6.5.2. The Quadrupole Ion trap

The quadrupole ion trap is based on the same theory as quadrupole analyser, with the electric fields generated within a three dimensional trap composed of a toroidal (ring) electrode, to which the RF voltage is applied, and two end cap electrodes creating the three dimensional electric field.

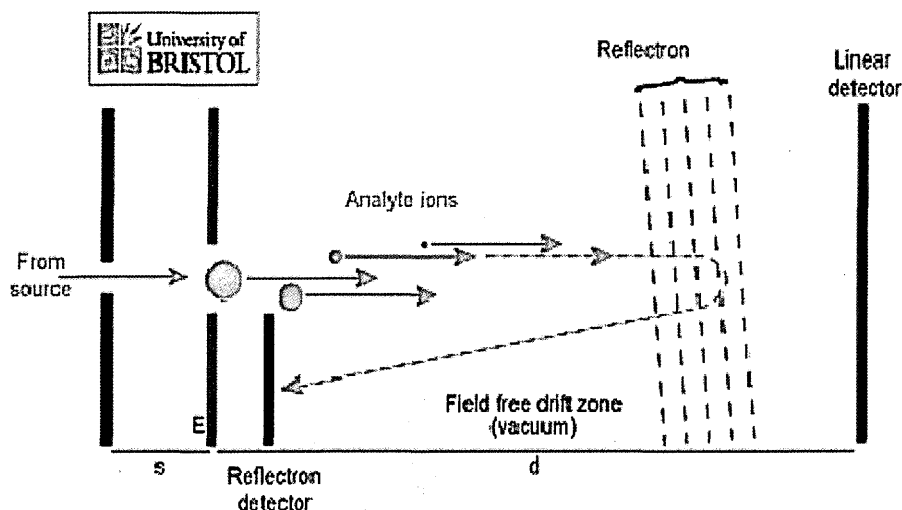


**Figure 14.** A schematic (cutaway view) of a Quadrupole Ion Trap Mass Analyser (from web source University of Bristol).

The trap is filled with helium and the ions in the trap describe mass-dependant orbital trajectories governed by the RF voltages and the cooling effects of collisions with the helium gas.

Mass spectra are acquired by sequentially ejecting ions from low  $m/z$  to high  $m/z$ , by scanning the RF voltage such that sequential ion trajectories become unstable. A product ion MS/MS spectrum is obtained by resonance excitation. The precursor ion is isolated by the application of a waveform signal to the endcap electrodes. Fragmentation of the precursor ion can be enabled by the application of a small voltage across the endcap electrodes.

#### 1.6.5.3. Time of Flight analysers



**Figure 15.** A Schematic of a Time-of-Flight mass spectrometer operating in Reflectron Mode (from web source University of Bristol).

In a time of flight (ToF) mass analysers, an accelerating voltage propels ions into a time of flight tube, where they drift towards a detector. Since ions arrive in the drift tube with similar kinetic energy (equal to  $1/2mv^2$ ), ions of different mass must have correspondingly different velocities, the heavier ions travelling more slowly. Measurement of the time of flight over the constant distance to the detector thus

enables calculation of velocity and so mass also, since flight time is proportional to the square root of the  $m/z$  value.

Theoretically mass resolution should be better with a longer flight tube, so ToF analysers often incorporate a reflectron (ion mirror) to reverse the trajectory of the ions back down the flight tube, effectively increasing the distance they traverse. Reflectrons also improve resolution by compensating for small differences in kinetic energy of ions of the same mass.

#### ***1.6.5.4. Q-ToF***

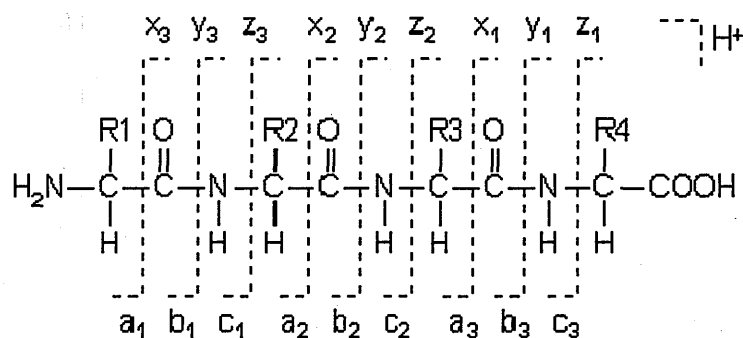
The Q-ToF is a hybrid instrument that combines a quadrupole mass filter with a reflectron ToF analyser, thereby providing an MS/MS capability. A collision cell, containing argon, is located between the quadrupole and the ToF analyzer to induce fragmentation (see below) in MS/MS experiments. The quadrupole, when operated in RF only mode acts as an ion guide allowing MS operation, and as mass selective device in MS/MS mode when the DC voltages are applied. The time-of-flight (ToF) analyzer is placed orthogonally to the quadrupole and serves as a mass resolving device for both MS and MS/MS modes. This provides high sensitivity, since ToFs, being non-scanning devices, detect all masses in the spectrum in parallel at high acquisition rates

#### ***1.6.6. Tandem Mass-Spectrometry significance***

Typically electrospray ionization produces little spontaneous fragmentation, so to obtain structurally informative tandem mass spectra (MS/MS) additional fragmentation must be induced artificially. Various dissociation methods to fragment ions are used, but gas phase collision-induced dissociation (CID) remains the commonest.

In a Q-ToF or ion trapping instrument, the process of CID involves multiple low-energy collisions of the peptide precursor ion with an inert gas, which converts translational energy into vibrationally excited states eventually resulting in bond cleavage. The fragments produced are the result of N-terminal and C-terminal fragmentations across the peptide bond to give sequence ions with charge retention on either the acylium fragment, which retains the original *N*-terminal residue (b-type ion) or ammonium (y) ion fragments retaining the C-terminus. (**figure 16**, "peptide nomenclature") [154].

Tryptic peptides by definition have readily protonated arginine or lysine residues at the C-terminus, which favours charge retention on the C-terminal containing fragment, so usually Y-type ions predominate in their low energy CID spectra, though internal lysine or arginines resulting from incomplete tryptic cleavage, or histidines towards the N-terminus, can increase the incidence of B-type cleavages. Instrument type and geometry can also exert a significant influence on detailed fragmentation patterns [155].



**Figure 16.** Fragmentation nomenclature.

Additional fragments attributable to loss of water or ammonia from these ions are commonly observed; thus b-ions can eliminate carbon monoxide forming a-ions. Immonium ions ( $\text{H}_2\text{N}=\text{CHR}$ , where R is the amino acid side chain) are also seen in the spectra as are internal fragments which contain neither the original N or C terminal residue.

### ***1.6.7. Protein identification and database searching***

The use of MS/MS and CID is becoming the accepted standard for protein identification and is steadily replacing peptide mass fingerprinting. The final stage is identification of proteins by searching the tandem mass spectra against a protein database. Computer search algorithms assign sequences to the lists of mass/intensity data from each acquired mass spectrum. These algorithms typically compare experimental mass spectra with theoretical ones generated *in silico* from peptide sequence strings in protein databases (*e.g.* Swiss-Prot/UniProt).

Scores are assigned using statistical and/or probabilistic methodologies (*e.g.* Sequest, Mascot, Phenyx) which provide an estimate of the likelihood of the match originating by chance alone. The sequence with the highest score is assigned as the identification. The identified peptides are then used to derive their parent proteins. Although different search engines process the same input spectra and often produce comparable outputs, subtle difference remain [156].

The Phenyx search engine was primarily used in these studies. This applies a two-stage analytical strategy (see **table 4** in Material and Methods section). The initial search typically uses stringent parameters with an extended database to obtain a filtered list of candidate proteins while minimising the computational overhead associated with too many combinatorial possibilities. In the second round a much more comprehensive search is performed on a limited database consisting only of the candidate proteins. This can encompass non-specific cleavages and consider a wide selection posttranslational and artefactual modifications. A search of a randomized version of the database enables estimation of the false-positive rate. The final

assignments and associated confidence levels calculated from the scores and the chosen database are then reported.



### **1.7. Pathway analysis**

The increasing body of data derived from global gene and protein expression measurements necessitates the use of computational technologies to store, analyze, interpret and learn from this information.

A number of different pathway databases and network building tools are now available, enabling the visualization of cellular components as networks of signalling, regulatory and biochemical interactions. Although most of these techniques were developed to accommodate the huge amount of data derived from gene microarray experiments, more recently they have been applied to proteomic data.

The interpretation of proteomic profiles is however a difficult task, as even simple stimuli can result changes in abundance of many proteins, although typically proteomic datasets are still smaller than those from gene microarray experiments. Many of the proteins that change in relative abundance may seem unconnected to the originally stimulated pathway. Thus, mapping of up- and/or down-regulated proteins onto known pathways, combining functional enrichment and transcription regulation analysis, might provide information to supplement the conventional approach of linking proteins to biological process via review of the literature. This may enable a richer interpretation of proteomic experiments and in the generation of new mechanistic hypotheses.

**CHAPTER 2**  
**AIMS OF THE THESIS**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be one of the most potent toxicants known and is the prototypical representative of the polyhalogenated aromatic hydrocarbon class of persistent environmental contaminants. Exposure to TCDD produces a wide variety of species- and tissue-specific effects including endocrine disruption [6, 10, 16, 157]. Recently, it has been suggested that endocrine disrupters could contribute etiologically not only to reproductive-related disorders but also to disorders involving non-reproductive tissues, as bone, whose homeostasis is tightly regulated by endocrine system [61]. While a broad range of dioxin toxicities have been well documented, its effects on bone are still poorly known. Only a few experimental studies have demonstrated that bone development and homeostasis are targets for TCDD [121, 158], although the mechanistic basis of this action is far from clear. Most of the studies on TCDD-induced changes in bone cells have been conducted on a protein-by-protein basis and a limited number of proteins [159, 160]. However the complex interactions among growth factors, hormones, cytokines, intracellular signalling pathways determining osteoprogenitor cell differentiation and bone remodelling call for more holistic view that can be provided by integrating a proteomic approach. Proteomic analysis offers great opportunities for its ability to focus on simultaneous changes of a large number of proteins, which can reveal the complex interplay of different pathways at a single time-point.

The main purpose of this study was to investigate the possible disturbance of TCDD on bone development, focusing on osteoblastogenesis, using a proteomic approach.

Toward this end, a model of rat mesenchymal stem cells differentiating to osteoblasts has been chosen, being a well-documented system for studying the molecular mechanism of osteogenesis *in vitro*.

The main objectives of this thesis were as follows:

- monitor the time-course progression of osteoblast differentiation using well known osteogenic markers and verify that the cell culture model was sensitive to TCDD and therefore useful for this study.
- determine whether TCDD affected the proteome profile of differentiating osteoblasts by using a global proteomic approach based on two-dimensional gel electrophoresis, computerized gel image analysis, in-gel digestion, and mass spectrometry
- determine how protein abundance changes evoked by TCDD exposure correlated with alterations in biochemical indices of osteoblast activity and differentiation, in an attempt to gain further insight into the molecular mechanism involved in TCDD's effect on osteoblast differentiation.

## **CHAPTER 3**

# **MATERIALS AND METHODS**

### 3.1. Reagents

2,3,7,8-TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was found to be over 99% pure by gas chromatography mass spectrometry. It was dissolved in dimethyl sulphoxide (DMSO). All chemicals and solvents used were of the highest purity available.

### 3.2. Mesenchymal stem cell isolation and osteoblast differentiation

Cell culture was performed in collaboration with KTL Institute, National Public Health Institute, Finland.

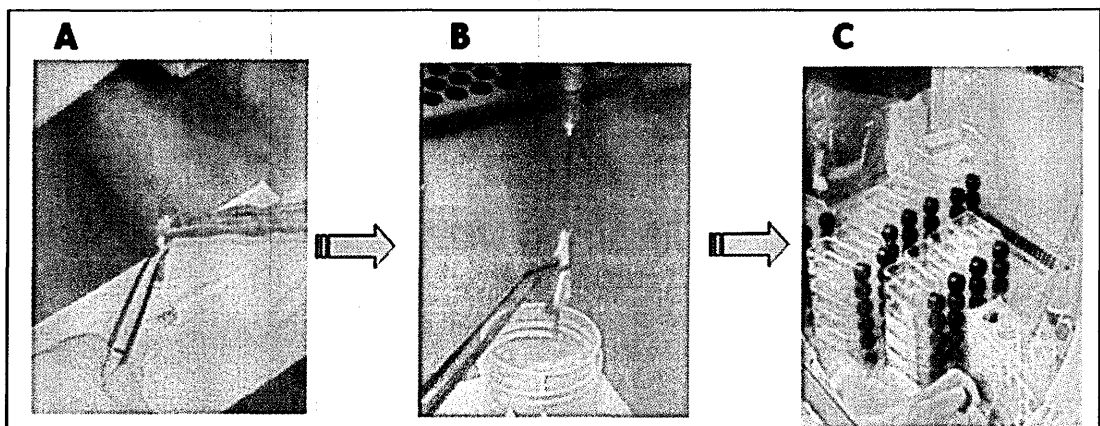
Mesenchymal stem cells (MSCs) were isolated from the bone marrow of femurs and tibias of male Sprague-Dawley rats, 6-weeks of age. The rats were purchased from Harlan (Zeist, The Netherlands) and maintained at the breeding colonies of the National Public Health Institute, Department of Environmental Health, Kuopio, Finland. Animal study protocols were reviewed and approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government. The animals were housed under controlled conditions with the temperature at  $21 \pm 1$  °C, relative humidity  $50 \pm 10\%$  and a 12 h lighting cycle. They had free access to tap water and standard pelleted laboratory animal feed R36 (Ewos, Södertälje, Sweden). MSCs for one experiment were collected from 4-5 animals.

After dissecting free of adhering tissues, bones were briefly soaked in ethanol and then in PBS (without calcium and magnesium).

As it is shown in **figure 17** the bone ends were removed aseptically in laminar hood and the marrow cavity was flushed out using alpha-MEM (Gibco [Invitrogen], Paisley, UK) medium supplemented with 7.5% fetal bovine serum (Gibco), 20 mM Hepes and

100 U/ml penicillin & 100 µg/ml streptomycin (Gibco). After homogenization by pipetting up and down, the suspension was incubated for 2-3 min in order to let the aggregates to settle down. Supernatant was collected and centrifuged at 210xg, 5 min. Cells were then resuspended into mineralization medium, which consisted of enriched alpha-MEM supplemented with 10 nM dexamethasone (Sigma, St. Louis, MO, USA), 10 mM glycerol phosphate (Sigma), and 50 µg/ml ascorbic acid (Wako Chemicals GmbH, Germany), which favour the differentiation of osteoblastic cells. Cells were counted by haemocytometer and plated at the density of  $1 \times 10^6$  cells/cm<sup>2</sup> in T25 cell culture flasks (Nunc, Roskilde, Denmark). The culture flasks were maintained in a humid atmosphere at 37°C with 5% CO<sub>2</sub>.

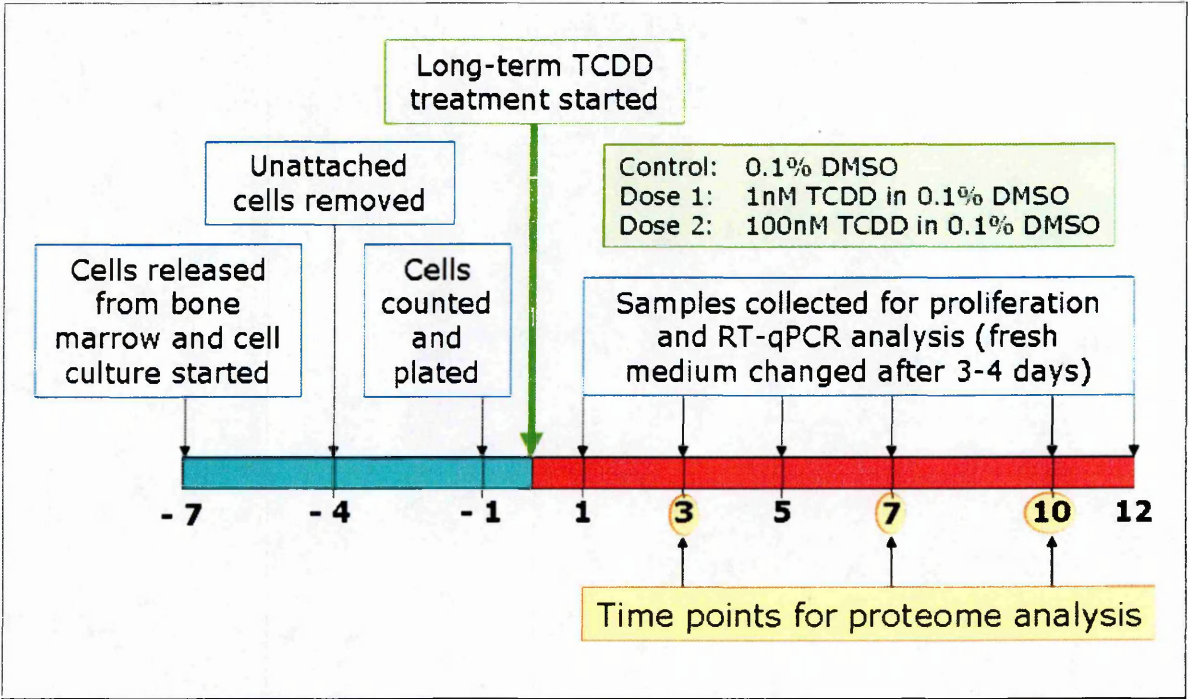
Three days later the non-adherent cells were removed by washing the attached cells with PBS. New mineralization medium was added. After reaching near-confluence (usually 6 days from the beginning of culture) the cells were detached with 0.075% trypsin-EDTA (Gibco). Cells were counted and plated into 6-well plates (45000cells/well) or 24-well plates (9000 cells/well).



**Figure 17.** Panel A: Sprague-Dawley male rat tibias were dissected free of adhering tissue. Bone ends were removed. Panel B: Marrow cavity was flushed out with HEPES-buffered completed alpha-MEM. Panel C: Released cells were plated.

3.3. Dioxin exposure

Next day the medium was replaced with exposure medium containing various concentrations of TCDD dissolved in DMSO (TCDD1: 1nM TCDD/0.1% DMSO; TCDD2: 100nM TCDD /0.1%), or DMSO vehicle alone so that the final concentration of DMSO was only 0.1%. The first day of TCDD exposure was defined as day 0. Thereafter, fresh exposure medium with mineralization ingredients was supplied to cells at every 3-4th day. Cells were harvested for different assays at timed intervals. The experimental design is shown in **figure 18**.



**Figure 18.** Experimental design



### **3.4. Sample collection**

Osteogenic induced samples were harvested at day 3-7-10 following TCDD exposure for proliferation assays, quantitative RT-PCR, Western blotting and proteomic analysis. Untreated cells and DMSO treated cells were harvested at the identical time points of osteogenic differentiation as control. Cells were collected by scraping, washed three times with ice-cold 10 mM PBS and pelleted. Pellets were shock-frozen in liquid nitrogen and stored at -80°C until further processing.

### 3.5. Cell proliferation and calcium quantification assay

For the cell proliferation test and the determination of calcium, osteoblasts were grown on 24-well plates. Cell proliferation was determined by colorimetric assay using Cell Proliferation Reagent WST-1 (Roche) directly to cells. The Cell Proliferation Reagent WST-1 provides a colorimetric assay for the quantification of adherent or suspension cell viability and proliferation. The reagent is a sterile, ready-to-use solution that contains WST-1 and an electron coupling reagent diluted in phosphate buffered saline. WST-1 is a tetrazolium salt that when in contact with metabolically active cells gets cleaved to formazan by mitochondrial dehydrogenases. After a short incubation, absorbance at 450nm indicated the rate of WST-cleavage by mitochondrial dehydrogenases, correlated with the number of viable cells in the culture. The darker the formazan dye, the greater the number of metabolically active cells in that well.

Briefly, medium was removed and WST-1 reagent was added to wells. Cells were incubated for 1 h. After shaking the cell plate for 1 min the media were transferred into 96-well-plate. The absorbance of the samples was measured using a plate reader at 450 nm (Labsystems iEMS Reader MF, Ascent Software version 2.4.1).

For calcium assay, the cells were washed twice with PBS and then fixed then in 3% PFA for 15-20 min. Calcium content was measured as triplicates by colorimetric assay using a Ca kit (Roche). Cells were washed twice with H<sub>2</sub>O and incubated overnight with 0.6 M HCl (on a shaker) using gentle shaking. The reagent mixture was added to the wells, incubated for 30 min, and absorbance measured at 546 nm using a plate reader.

### 3.6. Real time Quantitative PCR

Total cellular RNA was isolated from the pelleted osteoblasts using EZNA™ Total RNA Kit and RNase-free DNase set (Omega Biotek, Doraville, GA, USA). cDNA was generated by Omniscript RT Kit (Qiagen, Hilden, Germany) using random hexamers (Roche, Mannheim, Germany) and used as a template for quantitative PCR analysis. The expression levels of Runx2, Alp and Ocn were analyzed using SYBR Green PCR Master Mix kits and an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves were generated using isolated and purified PCR products produced with the same primers designed for quantitative PCR. PCR products were purified from agarose gels using QIAquick PCR Purification Kit (Qiagen) and the concentrations were determined spectrophotometrically.

PCR primers were designed using Primer Express software from Applied Biosystems that allowed the use of universal thermal cycling parameters. The following primers were used: rat GAPDH (NM-017008), atcccgcataacataaatgg and gtggttcacacccatcacaa; rat ALP (NM-013059), tgaatcggaacaacctgactga and ttccactagcaagaagaagccttt; rat OCN (NM-013414), aagcccagcgactctgagtct and ccggagtctattcaccaccttact; rat RUNX2 (XM-346016), ctccaacccacgaatgcacta and gtgagtgggtggcggacatg.

Each PCR reaction was initiated with an incubation step of 10 min at 95°C to activate AmpliTaq Gold DNA Polymerase. This was followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was run to confirm the absence of non-specific amplification. Negative controls were included in each run. The expression levels were related to mRNA concentrations of the housekeeping gene GAPDH to normalize the amount of cDNA in the PCRs.

The experiments were repeated independently three times using three replicates. Statistical analysis was performed by the ANOVA followed by the Least Significant

Difference test. In the case of non-homogenous variances the Mann-Whitney U test was used. The limit of statistical significance was set at  $p < 0.05$ .

### 3.7. Protein extraction for 2D-Gel electrophoresis

#### *3.7.1. Set-up of protein extraction protocol*

In this project, rat mesenchymal stem cell along the osteoblast differentiation pathway has been chosen as a model system to compare the proteins expression between untreated and TCDD-exposed osteoblasts. It has to be underlined that differentiating osteoblasts are particularly difficult to grow. Consequently, one of the main constraints I had to face was the limited number of cells to work with. The set-up of an efficient protein extraction procedure was therefore mandatory.

I decided to start with some non-bone cell lines that, due to their immortalized nature, are easy to grow, to expand and can easily provide enough biological material to start with. I used a proteomic approach on two cell lines available in the laboratory (the human lung adenocarcinoma cell line A549 and the human bladder carcinoma cell line RT112), in order to optimise sample preparation strategy and to maximize the number of proteins extracted from the minimum amount of cells.

Various types of detergents and lysis buffer composition were employed to optimize the efficiency of the lysis and the protein solubilisation (**table 3**). To this purpose, urea and the zwitterionic detergent CHAPS were used at different molarities. Thiourea was used to improve solubilisation of membrane proteins. We found that the addition of the Zwittergent detergent further improved the extraction of more hydrophobic proteins. The reducing agent such as DTT was used to break any disulphide bonds and to maintain all proteins in their fully reduced state. This is not easily achieved because DTT (pKa 8.2) is lost from the basic part of the strip during the IEF run. A solution to this problem has been found adding the Destreak (bis-hydroxyethyl disulfide, GE Healthcare, Italy) reagent to the rehydration buffer before the IEF run, instead of DTT in lysis buffer. Destreak reagent modifies the proteins by binding thiolhydroxyethyl

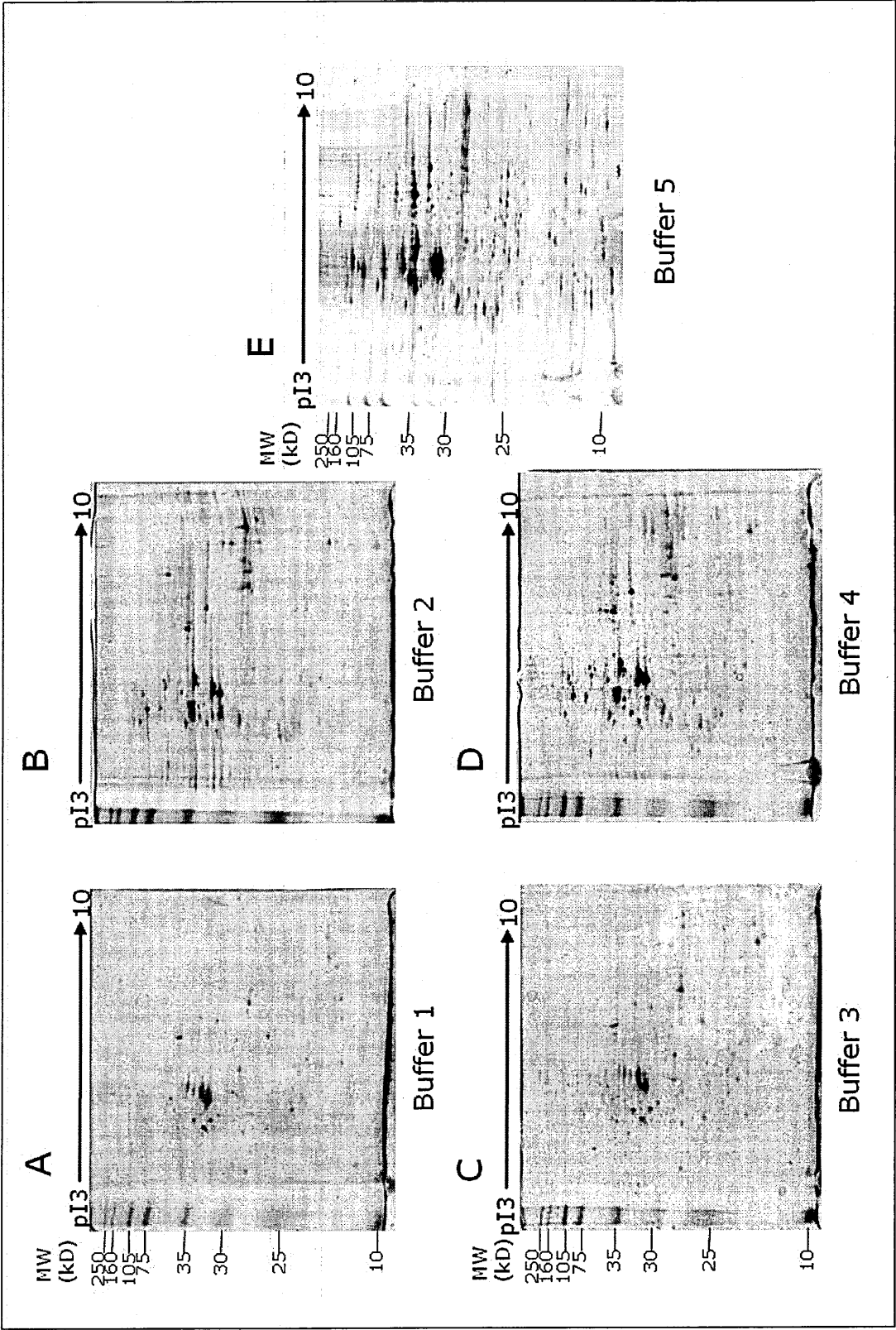
groups to cysteinyl groups resulting in time-stable, 2D maps with improved reproducibility.

**Table 3.** Composition of different lysis buffers used.

<b>Buffer 1</b>	8M urea, 2% CHAPS, 100 mM DTT
<b>Buffer 2</b>	7M urea, 2M thiourea, 2% CHAPS, 100 mM DTT
<b>Buffer 3</b>	8M urea, 4% CHAPS, 100 mM DTT
<b>Buffer 4</b>	5M urea, 2M thiourea, 2% CHAPS, 2% Zwittergent, 100 mM DTT
<b>Buffer 5</b>	5M urea, 2M thiourea, 2% CHAPS, 2% Zwittergent

**Figure 19** shows the Colloidal Coomassie Blue-stained 2-DE gels of whole cell lysate (200 µg) obtained using different extraction buffers.

As shown in **figure 19**, the use of buffer 5 provided the most informative 2-DE gel map.



**Figure 19.** Colloidal Blue Coomassie-stained 2-DE gels of cell protein extract (A549 cell line) obtained using different buffer compositions (see table 3).

***3.7.2. Application of the optimized protein extraction protocol***

Osteogenic induced samples were harvested at day 3, 7 10 following TCDD exposure. Untreated cells were harvested at each time point of osteogenic differentiation as control. Cells were collected by scraping, washed three times with ice-cold 10 mM PBS and pelleted. Pellets were shock-frozen in liquid nitrogen and stored at -80°C until further processing. Cell lysis and protein extraction was done using buffer 5 containing 5M urea, 2M thiourea, 2% CHAPS, 2% Zwittergent and a mixture of protease inhibitors (complete, mini EDTA-free cocktail, Roche). DNA was sheared by passing the lysate through a 21G½-gauge needle of a 1 ml syringe. Th lysate was sonicated for 3 minutes and then incubated for 40 minutes at 4°C on a mixing wheel. Cell debris was removed by centrifugation at 15,000xg for 15 min, then the supernatant was aliquoted and stored at -80°C until further processing.



### **3.8. Protein quantification for two-dimensional gel electrophoresis**

Proteins extracted from total cell lysate for proteomic analysis were quantified using PlusOne 2-D Quant Kit (GE Healthcare, Italy). This kit is designed for the accurate determination of protein concentration in samples to be analyzed by 2-D electrophoresis. Many of the reagents used in the preparation of such samples, (*e.g.* detergents, reductants, chaotropes and carrier ampholytes), are incompatible with the majority of commercially available protein quantification kits.

The PlusOne 2-D Quant Kit works by quantitatively precipitating proteins while leaving interfering substances in solution. The assay is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0-50  $\mu\text{g}$ . The absorbance of each sample and standard is read at 480 using water as reference.

### 3.9. Two-dimensional gel electrophoresis (2-DE)

For each gel, 200 µg of total protein were dissolved to a final volume of 250 µL in the re-hydration solution (5M urea, 2M thiourea, 2% CHAPS, 2% Zwittergent, 100 mM DeStreak and 0.5% IPG buffer pH 3-10 linear, GE Healthcare, Italy), and then applied on 13-cm-long immobilized pH 3-10 linear gradient strips (IPG strip, GE Healthcare, Italy). The strips were hydrated on an IPGphor apparatus (GE Healthcare, Italy) for 16 h at 30 V/h, then focused for 26 h until 46,000 Vhr, according to the following program: rehydration 15h at 30V, 2h at 200V, 1h at 500V, 30min at 1000V, 1h of a linear gradient to 8000V, 5h at 8000v. After the first-dimension run, proteins were reduced by incubating individual strips for 15 min in a solution containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 60 mM dithiothreitol (DTT, GE Healthcare, Italy). Proteins were then alkylated by incubating the strips for 15 min in a similar solution, with DTT replaced by 100 mM iodoacetamide. The strips were embedded in 0.7% (w/v) agarose on the top of 1 mm-thick acrylamide gels cast at gradient (7.5-17.5 %). Proteins were separated by mass using electrophoresis at 10 mA/gel. This was done overnight, at 4°C, in a running buffer composed of 25 mM Tris, 250 mM glycine, 0.1% SDS. Gels were rinsed three times with de-ionised H<sub>2</sub>O, fixed for 1 hour in an aqueous solution with 50% methanol and 7% acetic acid, and rinsed three times again with de-ionised H<sub>2</sub>O. Finally, gels were stained with colloidal Coomassie Blue (Pierce) for 4-5 hours then extensively washed with deionised H<sub>2</sub>O. Colloidal Coomassie blue reagent utilizes the colloidal properties of coomassie G-250 dye for protein staining on polyacrylamide gels. This unique reagent stains only protein and allows bands to be viewed directly on the gel during the staining process. It is more sensitive than traditional blue coomassie staining and it is compatible with mass spectral analysis.

Four replicate gels were run for each experimental condition (untreated and 2 doses TCDD-treated cells).

### **3.10. Two-dimensional gel electrophoresis gel image analysis**

Stained gels were scanned at 16-bit and 240 dpi resolution (Expression 1680 Pro, Epson) using same settings each time a gel was scanned and the resulting TIFF images were analyzed with two different image analysis packages: Progenesis PG240 software (v2006, Nonlinear Dynamics, U.K., [www.nonlinear.com](http://www.nonlinear.com)) and Progenesis Same Spots software (v3-2008, Nonlinear Dynamics, U.K.). Unfortunately the second one was only available in the lab from the beginning of 2008.

#### ***3.10.1. Progenesis PG240 analysis***

The Progenesis PG240 automatic analysis protocol for the thirty-six images of the gels included spot detection, warping, background subtraction, average gel creation, matching and reference gel modification. Detection, warping, and matching of the protein spots were done using the "combined warp and match" algorithm, which uses nonparametric pattern recognition clustering technique to align different gel images. "Total spot volumes normalization" algorithm was used to calculate each protein spot volume as the sum of the intensities of the pixels within the spot's boundary, minus the background level within that same boundary ("Progenesis Background" algorithm), normalized to the total spot volumes in the gel. Spot detection occurred after the image was processed using the Intelligent Noise Correction Algorithm (INCA), which discriminates between true signal and high noise levels. INCA assesses noise at each pixel within an image and accurately removes this noise from all areas of the gel, including that within spot material. The final effect is a significant improvement in spot detection without the false positives, caused by data containing spikes, so it is possible to derive even small changes from noisy images. Spot INCA volumes were normalized

against the total volume of all the spots in the gel. Average gels were generated by the software for spot pattern comparison: they are a statistical combination of the replicate gels in a group, showing spot mean values with associated errors. The criteria for including a spot in the average gel were that any spot must be present in all replicates. Spot editing (spot splitting corrections and match editing) was done sparingly, and only on selected complex areas of the gel.

### ***3.10.2. Progenesis SameSpots analysis***

SameSpots gel image analysis consists of a fast and streamlined 2D workflow, which includes an innovative visual tool for highly advanced and automated image alignment. Here, images are first aligned by localised warping to an image selected as a master. Then spots are detected simultaneously across all the images leading to one spot map which is then overlaid on each warped gel image in the dataset leading to an identical spot outline across the gel series. After the alignment, SameSpots **automatic** analysis includes SameSpots detection, background subtraction, normalisation and matching. The result is perfectly aligned images at the pixel level, with 100% matching and no missing values, which radically reduces the need for editing spot detection and re-matching. This novel method attempts to reduce the subjectivity of user matching and address the issue of missing values which are a problem for data analysis as many techniques in multivariate analysis cannot be used with incomplete datasets. Results are then ready for validation and statistical analysis.

### **3.11. Proteomic statistical analysis**

Both univariate and multivariate statistical methods were used to analyse the data. Univariate methods, *e.g.* *Student's t-Test*, one-way ANOVA, are used to detect significant changes in the expression of individual proteins. Multivariate methods, *e.g.* principle components analysis, utilize all the protein spot data simultaneously, to look for patterns in abundance changes. The univariate approach gives strong candidates that have had significant changes in abundance, whilst the multivariate approaches can detect more subtle changes across sets of proteins that may be mechanistically correlated such as proteins in the same pathway. Both approaches have a role to play in data analysis. However, in this study the univariate approach was used primarily because it is currently the simplest to interpret conceptually and most commonly used in 2-DE gels differential expression analysis.

#### ***3.11.1. Univariate statistical approach***

Statistical comparisons of the individual protein abundance in the experimental groups (one-way ANOVA) and between-groups comparisons (multiple comparison test, Tukey Kramer HSD,  $p < 0.05$ ) were computed using JMP v6 software (SAS Institute Inc.). Differences between groups were considered significant when expression fold-change was  $\geq 1.5$  and  $t$  test  $< 0.05$ .

### **3.11.2. Multivariate statistical approach**

The multivariate statistical approach was performed by using *Progenesis Stats Package*, an advanced statistical analysis tool integrated in *SameSpots* software (Nonlinear dynamics, UK). A complete list of all the specifications of this software is available at the <http://www.nonlinear.com>. Principal component analysis (PCA) was applied in order to determine if samples have the groupings expected or if there are any outliers in the data. PCA is a multivariate statistical analysis that allows the representation of the original dataset in a new reference system formed by new variables called principal components (PCs) and provides a simplified graphical representation of the multidimensional data. The results are visualized in plot of protein variables (loadings) and plot of samples (scores). The *Stats package* includes also Correlation Analysis and Hierarchical Clustering based on expression profile data. Furthermore the software provides the q-value for each tested spots. The q-value controls the false discovery rate (FDR) where the focus is on achieving an acceptable ratio of true and false positives. For this analysis, the level of significance has been set up at  $FDR < 5\%$ .

### **3.12. Protein identification by tandem mass spectrometry**

Protein species with a significant modulation in their expression were excised manually from the gel under laminar flow sterilized hood to avoid keratin contamination.

Protein identification is based on the analysis of peptides generated by proteolytic digestion. To this purpose, the protease of choice is generally sequencing-grade modified trypsin, which hydrolyzes the protein on the C-terminal side of lysine and arginine, unless the subsequent amino acid in the sequence is a proline. This is advantageous as every peptide other than the protein C terminus has at least two sites for efficient protonation, the N-terminal amino group and the C-terminal basic residue, so peptides are readily ionized and detected as positive ions by mass spectrometry.

Two different in-gel digestion protocols were used: both manual procedure and robotic one.

Aliquots of the supernatant, containing tryptic peptides, were directly analyzed by tandem-mass spectrometry. Almost all protein identifications were performed using different types of mass-spectrometric platforms that were available in my lab at Mario Negri Institute (my Affiliated Research Centre) or during my stay in my supervisor Dr. Robin Wait's laboratory, at the Kennedy Institute, Imperial College, London.

#### ***3.12.1. Manual protein in-gel digestion***

The whole procedure is carried on working in a laminar flow sterilized hood, by wearing powder free gloves.

Briefly, excised spots were smashed into small pieces to increase the surface area of proteins/peptides in the solution for improved trypsin accessibility. The gel pieces were then washed with 100  $\mu$ L water and then dehydrated with 100  $\mu$ L acetonitrile twice.



Cysteine residues were reduced incubating gel pieces 30 minutes at 56 °C with Dithiothreitol (10 mM DTT/0.1 M  $\text{NH}_4\text{HCO}_3$ ). Spots were washed three times with acetonitrile and derivatized by treatment with Iodoacetamide (55 mM IAA/0.1 M  $\text{NH}_4\text{HCO}_3$ ).

Gel pieces were washed three times with 0.1 M hydrogen carbonate, dehydrated three times with 100% acetonitrile and dried in a SpeedVac centrifuge.

Digestion buffer containing sequencing-grade modified trypsin (12.5 ng/ $\mu\text{l}$  Roche, Switzerland, 50mM ammonium hydrogen carbonate) was added, and spots were incubated one hour at 4°C. When the digestion buffer was absorbed by the gel plug, extra trypsin-containing buffer was removed. Then buffer without trypsin was added to ensure that the gel plugs were kept wet during the digestion process. 5 mM  $\text{CaCl}_2$  was added in order to stabilize trypsin action, since calcium ion retards trypsin autolysis. In-gel protein digestion was performed over night at 37 °C.

Aliquots of the supernatant (approximately total digest volume was 20  $\mu\text{l}$ /sample) were directly analysed by mass spectrometry.

### ***3.12.2. Robotic protein in-gel digestion***

Protein in gel digestion was performed by an automatic digester (Genomic Solutions, Huntington, UK) at the Kennedy Institute, Imperial College, London. Excised spots were washed with 0.1 M ammonium hydrogen carbonate and with acetonitrile.

Cysteine residues were reduced with DTT and derivatized by treatment with iodoacetamide. Gel pieces were washed with ammonium hydrogen carbonate and dehydrated twice with acetonitrile and dried at 60 °C, prior to addition of modified trypsin (Promega, Madison, WI, USA; 10 L at 6.5 ng/l in 25 mM ammonium hydrogen carbonate).

Spots were incubated for eight hours at 37 °C with digestion buffer.

The products were sequentially extracted with 25 mM ammonium hydrogen carbonate, CH<sub>3</sub>CN, 5% formic acid, CH<sub>3</sub>CN twice and with 10% formic acid, acetonitrile twice to obtain a total volume of 200ul. Extracts were lyophilized and were re-dissolved in 10ul 0.1% formic acid. Aliquots of the sample were directly analysed by mass spectrometry.

### ***3.12.3. Liquid chromatography-ESI (electrospray ionization)- ion trap tandem mass spectrometry***

LC-MS/MS analysis was performed using an innovative microfluidic chip-based technology for nanoelectrospray, whose functionality is equivalent to conventional nanospray LC/MS, coupled to an ion trap mass spectrometer (Agilent 1200 LC/MSD Trap XCT).

To obtain concentration and separation of peptides, sample digest aliquots (6-8 µl, equal to one third of the total digest) were directly injected into a G4240-62001 HPLC-Chip (Agilent 1200 G4240A HPLC-Chip Cube), composed by a 40 nl enrichment column and a 75 µm x 43 mm separation column packed with Zorbax 300SB-C18 (5 µm) at a flow rate of 0.3 µl/min. The mobile phases consisted in 0.1% formic acid in water (eluent A) and 0.1% formic acid in CH<sub>3</sub>CN (eluent B). The linear solvent gradient was as follow: from 5% of B to 30% of B in 28 min, then to 90% of B in 15 min and finally re-equilibration to 5% of B for 10 min.

The electrospray source operated in positive ion mode, with the following conditions: capillary voltage, -1750 V; Cap exit voltage 100V, skimmer voltage, 30.0 V; dry gas flow, 3 L/min; dry temperature, 300°C. Data were acquired sequentially in MS mode (scan range of 200 to 2000 amu) and in data dependent mode, automatically recording the MS/MS spectra of the four most abundant ions in every scan cycle. The MS/MS

spectra were acquired with isolation width of 4 m/z and fragmentation amplitude of 1.3V.

Tandem mass spectra were analysed using the MS/MS search engine Phenyx version 2.1 (GenBio, Switzerland) ([www.phenyx-ms.com](http://www.phenyx-ms.com), [phenyx@genebio.com](mailto:phenyx@genebio.com)), against the UniProt\_Swiss Prot database (version 54.0). The search was enzymatically constrained for trypsin, and allowed for one missed cleavage site. Further search parameters were: no restriction on molecular weight (MW) and isoelectric point; taxonomy: *Rattus norvegicus*; fixed modification carbamidomethylation of cysteine; variable modification: oxidation of methionine.

**Table 4** concisely restates the main submission parameters including algorithm, scoring models, thresholds. In parallel, the spectra were also searched against the National Center for Biotechnology Information non-redundant (NCBI nr) database (4,076,784 sequences) using Mascot (Matrix Science).

For an identification to be considered valid we required that: (i) two or more peptides independently matched the same protein sequence, (ii) the peptide score was significant, typically greater than 55 ( $p < 0.05$ ), and (iii) the manual interpretation confirmed agreement between spectra and peptide sequence. In addition, Mascot searches of all spectra were performed against a randomized version of the NCBI database using the same parameters as in the main search. In no case did this search retrieve more than a single peptide, and in all instances the peptide score was below the 0.05 significance level.

**Table 4\_** Submission parameters to Phenyx MS/MS search engine.

Scoring model	HCTultra	
Parent charge	1,2,3 (trust=yes)	
Rounds	1	2
Modifications	Cys_CAM[fixed, all]	Cys_CAM[fixed, all]
	Oxidation_M[variable, none]	Oxidation_M[variable, none]
Enzyme	Trypsin_(KR_noP)	Trypsin_(KR_noP)
	miss. cleav.=1	miss. cleav.=2
	cleavMode=normal	cleavMode=half cleaved
AC score	8	8
Pept thresholds	score>=7.0	score>=6.0
	p-value<=1.0E-6	p-value<=1.0E-5
	length>=6	length>=6
Conflict resolution	none	yes
Parent tol.	1.0 Da	2.0 Da
Turbo scoring	tolerance=800.0ppm	no
	coverage >=0.2	
	series=b;b++;y;y++	

*Scoring model:* refers to the algorithm that generates the score between experimental and theoretical masses, depending on the type of instrument.

*Parent charge:* refers to the charge state of the parent ions, singly-, doubly-, triply-charged

*Rounds:* sets of calculations on the data. A second round of calculations is used to fine-tune the results from the first round of scoring because only the accession numbers that fulfilled the first round criteria are processed during the second round.

*Modifications:* fixed chemical/post-translational modification that occurs for every instance of the modifiable amino acid in the protein sequence; variable modification that may or may not occur.

*Enzyme:* the enzyme used to digest the protein. The error allowance for enzyme inefficiency is included as number of sites per peptide that were not cut (Miss Cleav) and as occurrence of the digestion according to the cleavage rules (one or both ends of protein) (CleavMode).

*AC score:* refers to the minimum significant value for a protein's accession number score. Protein matches scoring lower than this value (set at 6) are rejected from the identified proteins.

*Peptide thresholds:* refer to three parameters (i) minimum peptide z-score, the minimum distance from a random match; (ii) minimum peptide p-value, the probability of a peptide match in a database occurring by chance with the associated z-score or better. The lower the p-value, the more significant the match; (iii) minimum peptide length, peptides with less than the specified number of amino acids (6) are reported in the peptide match results but do not contribute to the protein score.

*Conflict resolution:* conflicts arise when the scoring algorithm can match more than one peptide with an acceptable z-score and p-value to a given spectrum. Phenyx resolves a conflict only if the conflicting peptide matches are of good enough quality, i.e. if they reach a minimum z-score and p-value. These thresholds are a function of the parent charge and are set by Phenyx. If the zscores and p-values are too low, then the conflict is not resolved and the matches are rejected.

*Parent tolerance:* parent error tolerance, the deviation allowed between experimental (observed) parent ion masses and the theoretical (calculated) masses.

*Turbo scoring:* a procedure that accelerates the searches by pre-processing the data before submitting them to the main scoring calculation. A minimum percentage (20%) of the peptide sequence coverage by b+(b), b2+ (b++), y+ (y) or y2+ (y++) fragment series is looked for. If this percentage is not attained, the spectrum is not submitted for further scoring.

In-depth explanations of each descriptor are available at [www.phenyx-ms.com/](http://www.phenyx-ms.com/)

**3.12.4. Liquid chromatography-ESI (electrospray ionization)-quadrupole-Time o flight mass spectrometry**

LC-qToF analysis was performed at the Kennedy Institute, Imperial College, London.

Tandem mass spectra were recorded using a Waters Q-ToF instrument (Waters, Manchester, UK) interfaced to a Waters CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, injected onto a PepMap C18 column (300 μmx

0.5 cm; LC Packings, Amsterdam, The Netherlands), and eluted with an acetonitrile-0.1% formic acid gradient (5–70% acetonitrile over 20 min) at a flow rate of 1  $\mu$ l/min. The capillary voltage was 3500 V. A survey scan over the  $m/z$  range 400–1300 was used to identify protonated peptides with charge states of 2, 3, or 4, which were automatically selected for data-dependent MS/MS analysis and fragmented by collision with argon. The resulting product ion spectra were transformed onto a singly charged  $m/z$  axis using a maximum entropy method (MaxEnt3, Waters), and proteins were identified by correlation of uninterpreted spectra to entries in Swiss-Prot/TrEMBL using ProteinLynx Global Server (Version 1.1, Waters). The database was created by merging the FASTA format files of Swiss-Prot/TrEMBL and their associated splice variants (1,768,175 entries at the time of writing). No taxonomic or protein mass and pI constraints were applied. One missed cleavage per peptide was allowed, and the initial mass tolerance window was set to 100 ppm. In parallel, the spectra were also searched using the MS/MS search engine Phenyx version 2.1 (GenBio, Switzerland) ([www.phenyx-ms.com](http://www.phenyx-ms.com), [phenyx@genebio.com](mailto:phenyx@genebio.com)), against the UniProt\_Swiss Prot database (version 54.0).

### ***3.12.5. Liquid chromatography-MALDI (matrix assisted laser desorption ionization)-quadrupole-Time of Flight mass spectrometry***

The identification of some proteins was further confirmed using a different kind of ionization (Maldi QStar, Applied Biosystems, USA) at Dr. Robin Wait's laboratory. Following digestion, 1  $\mu$ L of each extracted peptide sample was spotted together with 1  $\mu$ L 1% -cyano-4-hydroxycinnamic acid matrix (in 50% acetonitrile, 0.3%TFA) directly onto a MALDI target plate. The mass spectrometer was operated in the data-dependent mode.

### **3.13. Western Blot Analysis**

#### ***3.13.1. Protein extraction for Western blot analysis***

Cells extracts were prepared by lysing cells in the lysis buffer (Triton X-100, 1M Tris, 5M NaCl) in the presence of aprotinin, leupeptine and PMSF as protease inhibitors, for 40 minutes on ice. Insoluble materials were pelleted at 12,000xg for 10 minutes at 4°C and the protein concentration was determined using a Biorad assay kit (BioRad, Milan, Italy).

#### ***3.13.2. Protein quantification for Western blot analysis***

Proteins extracted from the Triton X-100 were quantified with a modified Bradford method [161] (Bio-rad Protein assay). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change.

#### ***3.13.3. Western Blot analysis***

Total cellular proteins (8 µg/lane) were separated on SDS-12% polyacrylamide resolving gels using the Mini Protean II electrophoresis system at 90V, for 2 hours (Bio-Rad, Milan, Italy). Proteins were transferred to nitrocellulose transfer membrane (Bio-Rad, Milan, Italy) using the transfer buffer (50 mM Tris, 100 mM glycine, SDS

0.01%, 20% methanol) and the Bio-Rad Trans-blot system (55V, 2h). Blots were rinsed with TBS-T buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% v/v Tween-20) and blocked in TBS-T, 5% w/v non fat dried milk (Nestlé, Italy) for 2 h. After overnight incubation with primary antibody diluted 1:400 in TBS-T, 5% non-fat dried milk, (rabbit polyclonal antibodies: Bcl2 N-19; Santa Cruz Biotechnology, USA; goat polyclonal antibodies: Annexin II A-15, Ran C-20, Calregulin N-19; Santa Cruz Biotechnology, USA; mouse monoclonal antibody: Vimentin clone V9, Sigma-Aldrich, USA; Bax B-9, Santa Cruz Biotechnology, USA; GAPDH 6C5; Calbiochem, Germany), blots were washed with TBS-T and incubated with secondary antibody at 1:1000 for 1h. Peroxidase-conjugated anti-mouse, anti-goat or anti-rabbit IgG HRP (Santa Cruz, Biotechnology, USA ) were used as secondary antibodies.

Blots were revealed using enhanced chemiluminescence (ECL) (GE Healthcare, Milan, Italy) visualized on autoradiography film (Hyperfilm ECL, GE Healthcare, Milan, Italy) and scanned as 14-bit images (Image Scanner II, GE Healthcare, Milan, Italy). The resulting TIFF images were analysed using the Progenesis software (v2006, Nonlinear Dynamics, U.K.). Expression data were normalized relative to GAPDH. Statistical comparisons of the individual protein abundance in the experimental groups (one-way ANOVA) and between-groups comparisons (multiple comparison test, Tukey Kramer HSD,  $p < 0.05$ ) were computed using Prism v.5.01 software (GraphPad, USA).

### 3.14. Confocal microscopy

Actin stress fibers ending to focal adhesions containing paxillin were visualized in cell cultures. The PFA-fixed, permeabilized (0.1 % (v/v) Triton-X-100 in PBS) and BSA treated (0.2 % BSA) cells were stained by using mouse monoclonal paxillin antibody (1/100; ZYMED Laboratories, San-Francisco, CA, USA) for 45 minutes on 4°C. Staining was carried out with secondary antibody (1/100; ALEXA Fluor 488 goat anti-mouse IgG, Molecular Probes, Oregon, USA) for 30 minutes at 4°C and with tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated phalloidin (T-PHD; Sigma Chemical Co., St. Louis, MO, USA) for 20 minutes at room temperature (Sigma Chemical Co., St. Louis, MO, USA).

Confocal microscope (Zeiss LSM 510), with Axiovert 100M microscope and 25mW Argon and 5mW HeNe lasers (Lasos Germany) with 63x objective (NA 1.2/water; Zeiss, Germany) was used to visualize the plane of attachment of the cells. The cover slips were mounted with glycerol PBS on objective glasses. For each culture well, 10 image frames (1024 × 1024 pixel frame size, pixel size 0.14 µm × 0.14 µm) were scanned from randomly chosen areas. The amount of paxillin and its organization in focal adhesions were visually evaluated from the acquired images.

This analysis was performed in collaboration with Prof. Juha Tuukkanen at the Department of Anatomy and Cell Biology, Institute of Biomedicine, University of Oulu, Finland.



### **3.15. Pathways analysis**

MetaCore (GeneGo St.Joseph, MI, USA) was used to map the differentially expressed proteins into biological networks and for functional interpretation of protein data. MetaCore is an integrated software suite, based on a manually curated database of human protein-protein, protein-DNA interactions, transcriptional factors, metabolic and signalling pathways.

The list of differentially expressed proteins was uploaded as respective Swiss-Prot IDs to MetaCore for analysis. The biological process enrichment was analyzed based on GO Ontology processes. For network analysis, the shortest path algorithm to map the shortest path for interaction was used. Generation of sub-networks centered on transcription regulation was further investigated to explore up-stream regulatory molecules as key-nodes. Networks were automatically ranked by a P-value and interpreted in terms of Gene Ontology. Details for the assembly and network analysis processes are available at <http://portal.genego.com>.

**CHAPTER 4**  
**RESULTS**

#### **4.1. Effect of TCDD on cell growth and differentiation**

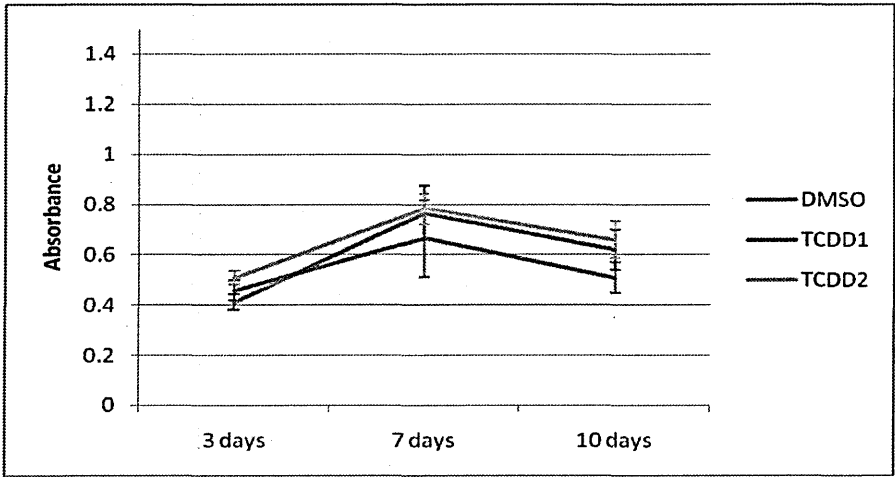
In this study, I exploited the ability of bone marrow stem cells (MSCs) to differentiate into bone-forming osteoblasts as a model system to study the effects of TCDD on developing bone.

MSCs were encouraged to differentiate to mature osteoblasts by the addition of dexamethasone, beta-glycerol phosphate and ascorbic acid, which are required, for the formation and mineralization of the extracellular matrix in the culture. As described in Section 1.3, differentiating osteoblasts progress through three phases: (i) an early proliferation period that continues for several days after confluence, with cells exiting the cell cycle between days 3 and 7, (ii) a period of collagen deposition and extracellular matrix maturation and (iii) terminal differentiation with mineralization of the mature matrix.

In this chapter of results, I describe how TCDD affects cell parameters and osteoblast activity in terms of mineralization and calcium deposition.

##### ***4.1.1. Cell proliferation and vitality***

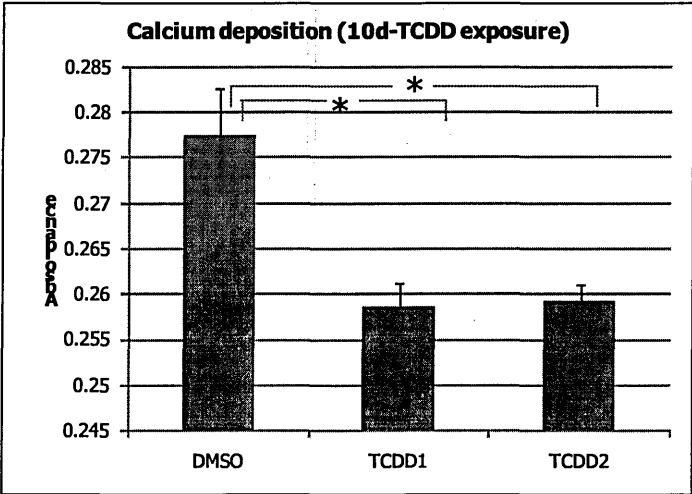
The condition of MSCs and their proliferation and differentiation were monitored frequently by microscopy. Cell proliferation was measured at several time points between three and thirteen days after TCDD exposure. After 7 days of culture, the proliferation rate of osteoblasts became slower, concomitantly with the progress of differentiation. Cells usually started to produce minerals around days 5-6, and eventually they became almost totally covered with minerals. As shown in **figure 20**, TCDD treatment at both doses (1 and 100nM) did not affect cell viability.



**Figure 20.** Effect of TCDD on time-dependent cell proliferation of differentiating osteoblasts derived from rat bone marrow. Cells were treated with 1nM TCDD (TCDD1) and 100 nM TCDD (TCDD2) dissolved in DMSO or DMSO alone on day 0 and samples were collected 3-7-10 days later. Each point represents the mean  $\pm$  SD of three replicates. Differences were not significant.

**4.1.2. Calcium deposition**

The calcium quantitation assay was performed exclusively on day 10 of exposure, since at this point untreated osteoblasts deposit enough calcium to be quantified. As shown in **figure 21**, the deposition of calcium by rat osteoblasts was significantly reduced by 1 nM TCDD and 100 nM TCDD.



**Figure 21.** Effect of 10 days of TCDD exposure on osteoblasts calcium deposition. DMSO, untreated cells, TCDD1: cells exposed to 1nM TCDD, TCDD2: cells exposed to 100nM TCDD. Each bar represents the mean  $\pm$  SD of three replicates. Asterisks highlight significant difference in calcium deposition levels (one-way ANOVA, Tukey Kramer HSD,  $p < 0.05$ ).

**4.1.3. Effect of TCDD on cell growth and differentiation: remarks**

The results indicated the usability of our *in vitro* model system utilizing differentiating osteoblasts when studying the effects of TCDD on bone development.

Cell viability and proliferation were not altered by TCDD exposure, even at the highest dose (100nM) for 10 days. However osteoblast functionality was altered since calcium deposition was inhibited by both TCDD doses.

#### **4.2. Effect of TCDD on time-dependent expression of osteogenic markers**

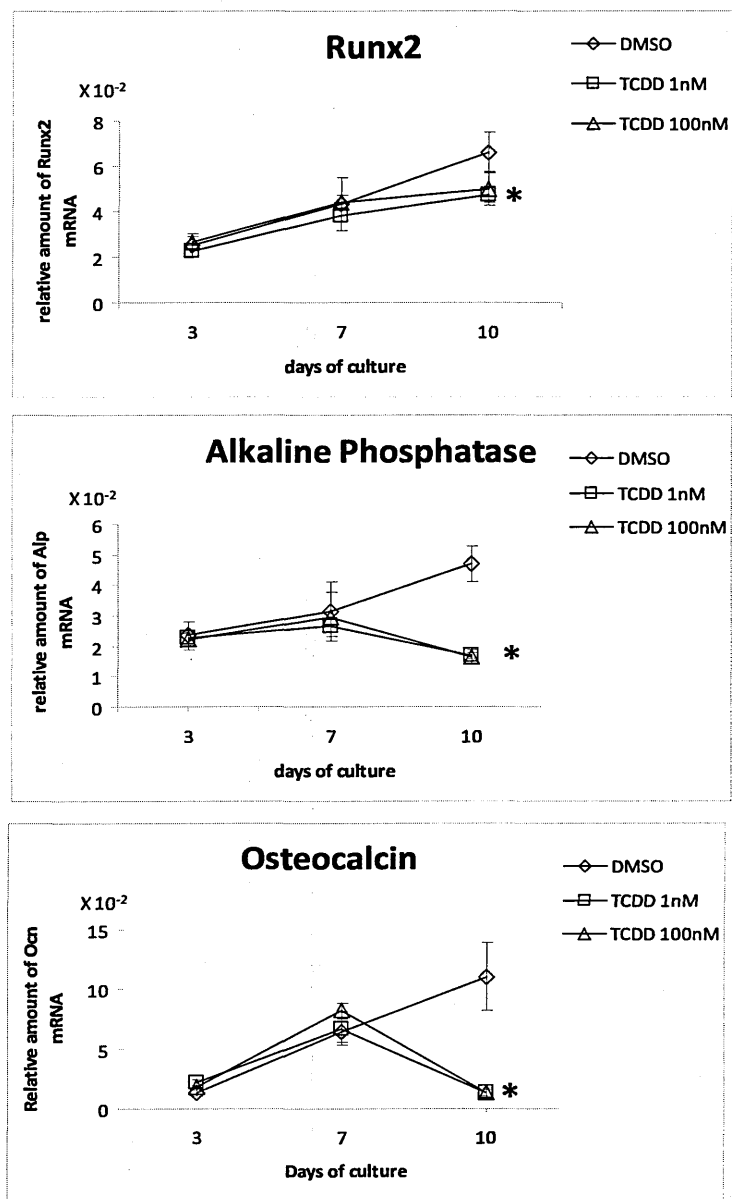
During osteoblast differentiation, the patterns of expression of typical markers are organized temporally and sequentially: the runt-related transcription factor 2 (Runx2) is expressed during active proliferation, alkaline phosphatase (Alp) during matrix maturation and osteocalcin (Ocn) at the onset of mineralization.

Thus, I monitored the progress of osteoblastic differentiation and the possible effect of TCDD by measuring the mRNA levels of Runx2 (marker for the proliferation step), Alp (marker for the matrix maturation step) and Ocn (marker for the mineralization step) using quantitative RT-PCR after 3, 7 and 10 days of culture in presence and absence of TCDD.

As shown in **figure 22**, Runx2, Alp and Ocn levels in the DMSO-treated cells were highest on day 10. Both TCDD doses (1 and 100 nM) did not have any effects on the markers level at 3 and 7 days of exposure, while at 10 days they reduced significantly the mRNA expression of Runx2, Alp and Ocn.

##### ***4.2.1 Effect of TCDD on time-dependent expression of osteogenic markers: remarks***

Expression of all measured marker characteristic for different stages of osteoblastic differentiation was dramatically reduced only after 10 days of TCDD exposure, indicating that TCDD significantly inhibits osteoblast differentiation *in vitro* in a dose-dependent manner only after 10 days of TCDD treatment.



**Figure 22.** Effect of TCDD on time-dependent mRNA expression of Runx2, Alkaline Phosphatase (Alp) and Osteocalcin (Ocn) in differentiating osteoblasts derived from rat bone marrow. Cells were treated with 1nM TCDD (squares) and 100 nM TCDD (triangles) dissolved in DMSO or DMSO alone (diamonds) on day 0 and samples were collected 3-7-10 days later. Each point represents the mean  $\pm$  SD of three replicates. Statistically significant differences induced by TCDD vs. control are depicted with asterisks (one-way ANOVA, LSD test,  $p < 0.05$ ).

### 4.3. Differential proteomic analysis using univariate approach

To further investigate the disturbance of osteogenesis caused by dioxin, I adopted a proteomic approach to examine the overall protein expression profile in differentiating osteoblasts and to characterize alterations evoked by TCDD.

Differentiating osteoblasts, exposed to various concentrations of TCDD (1 and 100nM) were analyzed at the same time points (3, 7 and 10 days exposure) as mRNA levels of osteogenic markers. As reported in section 4.2, during the three main phases of osteogenesis, the mRNA expression levels of typical osteogenic markers such as Runx2 (proliferation), Alp (matrix maturation) and Ocn (mineralization) decreased dramatically after 10 days of exposure to TCDD. This prompted me to investigate *first* the proteome profile change at this time point, at which the anti-osteogenic potential of dioxin was observed. *Then* I focused my attention on earlier time points of exposure (3 and 7 days), at which no effect on sequential differentiation markers expression was found. Differential protein expression was assessed using two-dimensional gel electrophoresis, computerized gel image analysis, in-gel digestion, and tandem-mass spectrometry.

In this chapter of results, I will report the comparative proteomic analysis performed using a *univariate statistical method* to detect statistical changes in the expression of individual proteins.

Although the enormous number of variable measured in proteomics (spots) and the relatively small samples number would call for a different data analysis (exploratory data approach), the univariate analysis is currently the simplest to interpret conceptually and the most commonly used in 2-DE gels differential expression analysis (see section 1.4.2.3).

Proteome profiles at each time point have been analysed separately, comparing the individual protein abundance in TCDD treated samples with their untreated counterpart



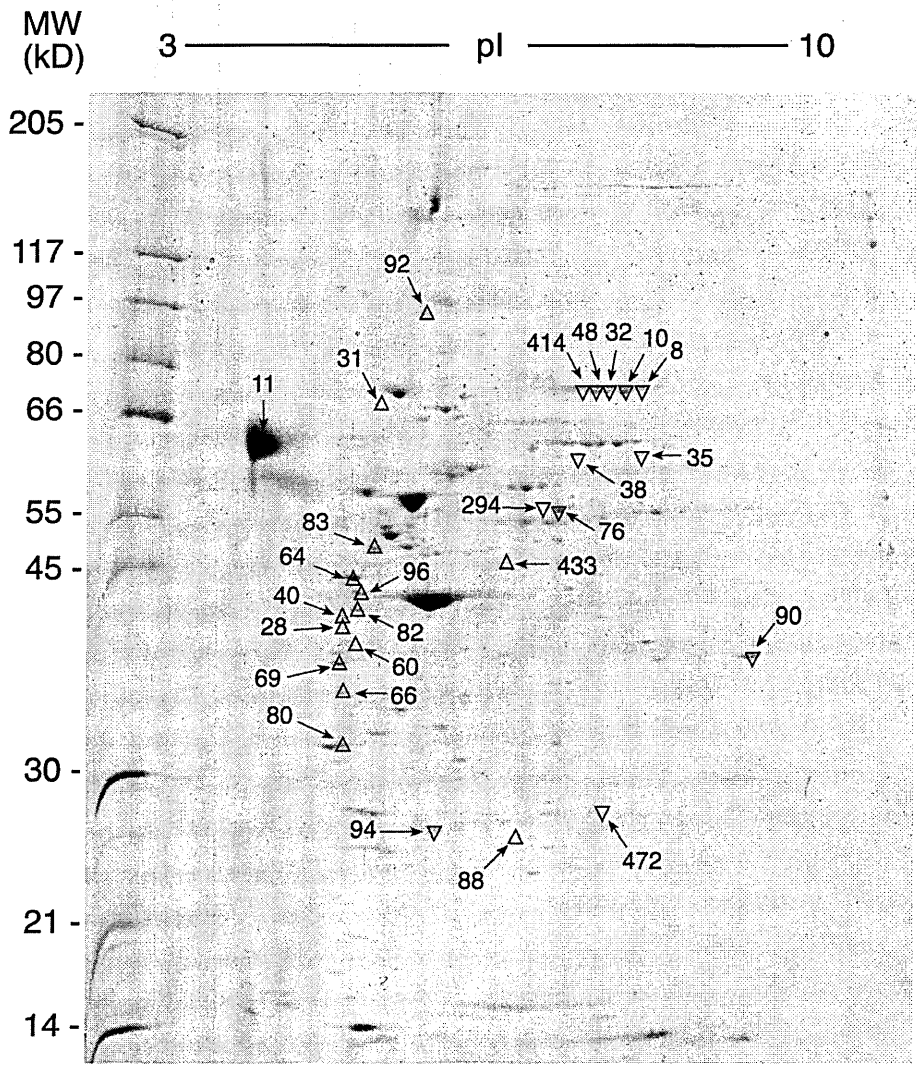
using one-way ANOVA and correcting for multiple comparisons (Tukey Kramer HSD test).

#### **4.3.1. Proteome profile of differentiating osteoblasts exposed for 10 days to TCDD**

The protein expression pattern of differentiating osteoblasts exposed to TCDD for 10 days was compared with their untreated counterpart using 2-DE image analysis software.

**Figure 23** shows a typical 2-DE gel representative of unexposed osteoblasts (averaged from four replicates). Triangles represent protein species with lower (apex down) or greater (apex up) expression in TCDD-exposed osteoblasts with respect to unexposed osteoblasts.

Overall, 27 protein species, out of 790 visualised, showed a statistically significant abundance change of 1.5 times or more, as a result of TCDD exposure. Results of MS/MS identifications are summarized in **table 4**. Every protein is validly identified by at least one of the instrument. Detail information on peptide/protein identification is reported in **supplemental table 1** (Appendix 7.3 p. 202).



**Figure 23.** Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate (200 µg proteins) from unexposed osteoblasts (DMSO). The average gel generated by image analysis of four replicates samples is shown. Triangles represent protein species with lower (down) or greater (up) expression in 10 days TCCD-exposed osteoblasts compared to unexposed osteoblasts. Spot numbers result from the Progenesis image analysis.

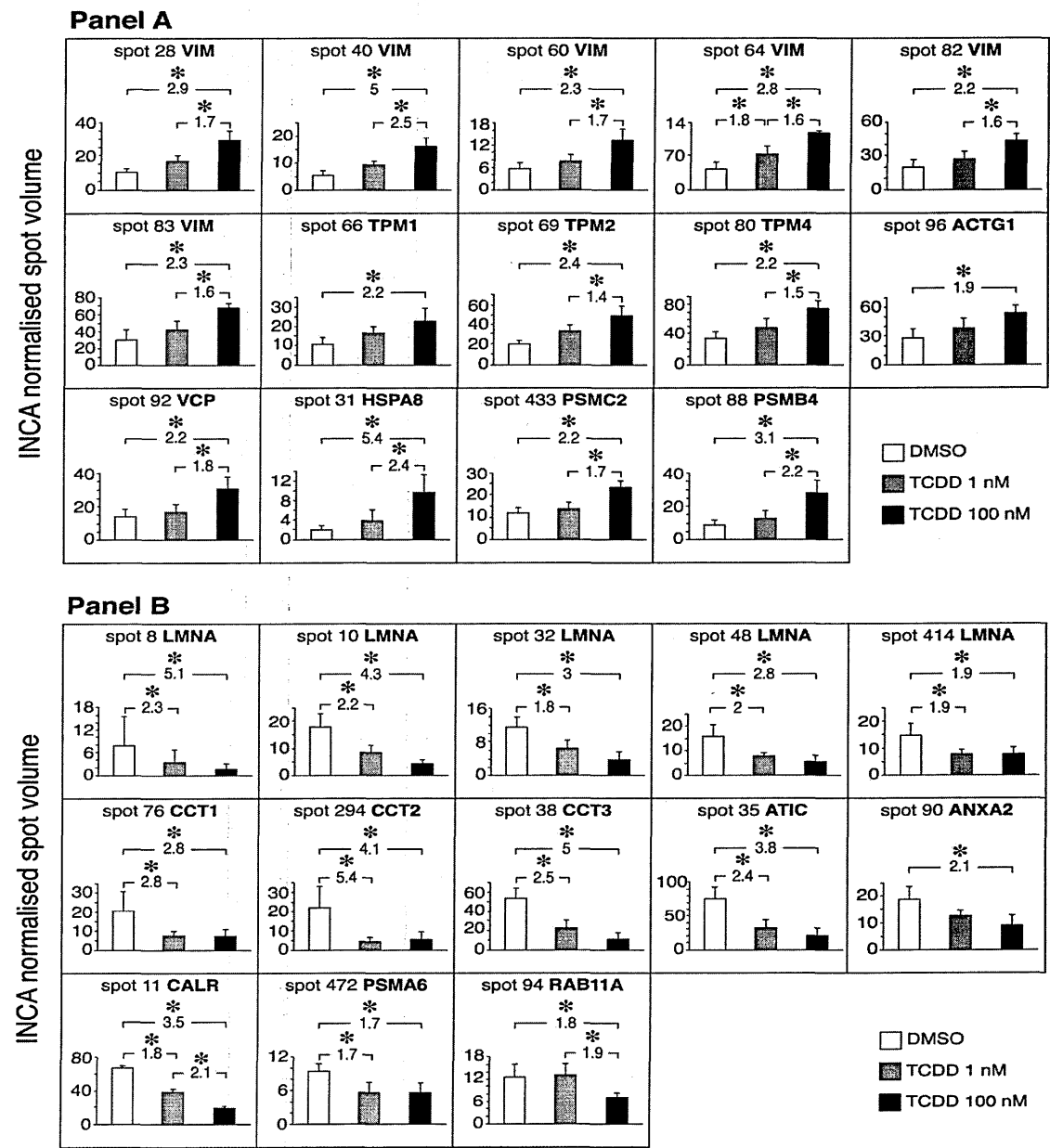
**Table 5.** Identification of proteins showing different levels of expression in untreated and 10 days TCDD-treated rat osteoblasts, by 2 different LC-MS/MS platforms. Detailed information on peptide/protein identification is reported in **supplemental table 1** (Appendix 7.3).

Spot #	Identified protein	Symbol	Uniprot-Swiss-Prot #	pI Theor./Ex p. a)	MW (kD) Theor./Ex p.a)	LC-ESI-IT			LC-ESI-Q-TOF		
						# unique pept. b)	Cov% c)	Score d)	# unique pept. b)	Cov % c)	Score d)
8	Lamin-A	LMNA	P48679	6.7/7.7	74.3/70	25	42	258.6			
10	Lamin-A	LMNA	P48679	6.7/7.5	74.3/70	15	25	139	7	12	59.3
11	Calregulin	CALR	P18418	4.4/4.2	46.3/60	16	53	131.8	9	34	91.4
28	Vimentin	VIM	P31000	5.07/4.9	54/37	34	57	362.3	15	33	132.4
31	Heat shock cognate 71 kDa protein	HSPA8	P63018	5.4/5.3	70.9/67	29	47	287.7	15	33	151.6
32	Lamin-A	LMNA	P48679	6.7/7.3	74.3/70	6	11	63.2	2	3	18.5
35	phosphoribosylaminoimidazolecarboxamide formyltransferase	ATIC	O35567	6.9/7.7	64.2/64	7	19	65.3			
38	T-complex protein 1 subunit gamma	CCT3	Q6P502	6.4/7.1	60.6/64	2	4	20.1	4	7	35.1
40	Vimentin	VIM	P31000	5.07/4.9	54/38	29	51	314.2	13	38	145.5
48	Lamin-A	LMNA	P48679	6.7/7.1	74/70	15	28	150.4			
60	Vimentin	VIM	P31000	5.07/5	54/35	20	41	191.8	10	17	86.9
64	Vimentin	VIM	P31000	5.07/5	54/44	68	73	775.4	16	36	157.9
66	Tropomyosin 1 alpha chain	TPM1	P04692	4.78/4.8	33/32	28	70	277.6	6	25	57.9
69	Tropomyosin beta chain	TPM2	P58775	4.65/4.8	33/34	34	80	335.5	13	40	120.8
76	T-complex protein subunit alpha	CCT1	Q5XIM9	6.09/6.7	57.4/57	14	30	135	13	35	145.6
80	Tropomyosin alpha-4 chain	TPM4	P09495	4.69/4.7	28/31	29	73	270.2	12	57	98.6
82	Vimentin	VIM	P31000	5.07/5	54/39	46	60	479.3	12	27	120.9
83	Vimentin	VIM	P31000	5.07/5.1	54/50	62	72	478.2	15	36	159.6
88	Proteasome subunit beta type 4	PSMB4	P34067	6.3/6.3	24.4/25	5	27	46.7	5	22	53.6
90	Annexin II	ANXA2	Q07936	8.1/8.5	38.8/40	8	22	75.2	13	49	129.2
92	Valosin-containing protein	VCP	P46462	5.17/5.7	89/95	45	56	490.4	24	47	233.8
94	Ras-related protein Rab-11A	RAB11A	P62494	5.6/5.8	22.8/25	2	12	18.9			
96	Gamma-actin	ACTG1	P63259	5.4/5.2	41.8/43	22	46	197.4			

294	T-complex protein 1 subunit beta	CCT2	Q5XIM9	6.1/6.8	57.5/57	6	12	13	35	147.9
414	Lamin-A	LMNA	P48679	6.7/7	74/70	12	20	3	5	23.4
433	26S protease regulatory subunit 7	PSMC2	Q63347	5.7/6.5	49/49	7	19	5	14	41.1
472	Proteasome subunit alpha type 6	PSMA6	P60901	6.8/7.2	27.4/28	3	13	8	24	79.2

- a) Theor.: theoretical, data-based annotations; Exp.: experimental, from 2-D gels.
- b) # unique pept.: number of unique peptide matches found for the given protein.
- c) Cov%: all amino acids from valid peptide matches as a percentage of the total number of amino acids in the protein.
- d) Score: the protein score is calculated from the individual normalized z-scores of validated peptides. Peptide z-score refers to the distribution of calculated scores compared to that of random peptide sequences in order to find the mean and variance ([www.phenyx-ms.com](http://www.phenyx-ms.com)).
- Database redundancy is handled and solved by the Phenyx software. If a protein shares all of its validated peptides with another protein, then it is considered to be a subset and will not appear in the best-scoring protein list. It appears in the protein Details panel under Subset for the principal and better scoring parameters. Therefore, the entries reported refer exclusively to the best-scoring protein found by the search engine.

**Figure 24** provides an overview of the expression patterns of the 27 protein species, whose abundance changed significantly in differentiating osteoblasts exposed to TCDD doses for 10 days.



**Figure 24.** Expression patterns for the selected proteins whose abundance changed significantly in differentiating osteoblasts unexposed (DMSO) and exposed to two doses of TCDD (1 and 100 nM). Each bar represents the average spot abundance expressed as INCA normalised volume  $\pm$  SD. The vertical axis shows the INCA normalised spot volume. Numbers indicate the difference in protein expression (-fold). Asterisks highlight significant expression changes (one-way ANOVA, Tukey Kramer HSD  $p < 0.05$ ). Spot numbers are from the Progenesis image analysis results. Panel A: proteins up-regulated after ten days of TCDD exposure. Panel B: proteins down-regulated after ten days of TCDD exposure. Spot numbers refer to **figure 23**.

#### 4.3.1.1. Proteins up-regulated following 10 days of TCDD exposure

TCDD exposure, mainly at the highest dose (100nM), significantly boosted the expression of 14 gel features (**figure 24, panel A**), which correspond to 9 individual proteins (**table 5**).

At least 6 spots were identified by MS/MS as vimentin (spots # 28, 40, 60, 64, 82, 83), a type III intermediate filament. Changes in vimentin expression have been reported in response to oxidative stress, suggesting a role for the vimentin network under condition of stress [162-164] and its involvement with signalling molecules [165, 166]. Some vimentin forms (spots # 28, 40, 60, 64, 82) had a smaller than the theoretical molecular weight, which would indicate proteolysis of the protein. The presence of such truncated forms might be compatible with the reported cleavage products of vimentin during the early phase of apoptosis [167]. Using tandem-mass spectrometry, I searched for vimentin-specific peptides resulting from cleavage at the two major caspase- sites at, Asp<sup>85</sup> (caspase-3) and Asp<sup>259</sup> (caspase-8)[167, 168]. I found multiple peptide coverage spanning 15 residues 78-400 for almost all of the six vimentin isoforms (**figure 25**). The most *N*-terminal of these peptides started at residue 70-78 of the intact protein, and included the putative caspase-3 cleavage site at residue 85. No peptide was identified including the possible second cleavage site at residue 259, but multiple peptides were abundantly found downstream. Thus, as the peptide coverage was very similar for each of the isoforms, I was unable to find evidence of protease cleavage leading to the specific vimentin forms I observed.

Interestingly, further cytoskeletal components including tropomyosins (TPMs) were up-regulated in differentiating osteoblasts after TCDD exposure. Tropomyosins are a large class of actin binding proteins that regulate microfilament stability and organization by recruiting other regulatory proteins to actin, or alternatively, by inhibiting their binding [169]. Tropomyosins are derived from four genes (alpha, beta, gamma, and delta) and over 20 isoforms can be generated by alternative splicing. In my osteoblast population,

the highest dose of TCDD significantly enhanced the expression of TPM1, TPM2 and TPM4 isoforms (spots # 66, 69, 80), approximately doubling them compared to untreated osteoblasts.

The expression of a further component of the cytoskeleton, gamma-actin (ACTG1, spot #96), was also almost doubled by the high TCDD dose. The cytoskeleton beta- and gamma-actin isoforms are ubiquitous in the microfilament system and it has been reported that over-expression of the beta-isoform in myoblast causes a striking increase in cell motility[170]. It is not yet clear whether this might be the case also for overexpression of the gamma-isoform. It is also not known how the expression of beta- and gamma-actin coordinates cell structure and functions.

The highest dose of TCDD induced the expression of valosin-containing protein (VCP, spot #92), a hexameric type II ATPase of the AAA family (ATPases with multiple cellular activities) that influences various cell functions, including endoplasmic reticulum-associated degradation (ERAD) through the ubiquitin-proteasome system (UPS) [171].

I also observed a statistically significant increase in the expression of the heat shock cognate 71-kDa protein (HSPA8, spot #31), a cytosolic chaperonin whose abundance increased 5 fold following the high dose of TCDD.

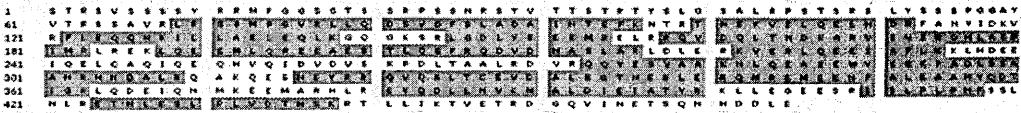
The high dose also caused significant up-regulation of two proteasome subunits: PSMC2 (spot # 433), the regulatory subunit of the 26S proteasome with ATPase, which interacts with several basal transcription factors [172] and PSMB4 (spot #88), a beta-type subunit of the 20S proteasome (2.2 and 3.1-fold, respectively).

(a)

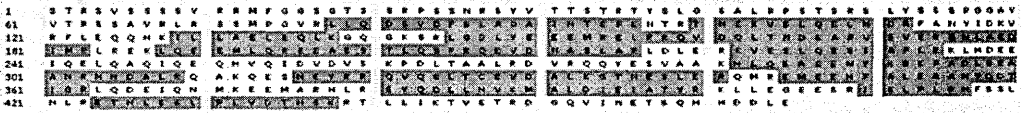
MSTRSVSSSSYRRMFGGSGTSSRPSSNRSYVTTSTRTYSLGSAALRPSTSRSLYSSSPGGAYVTRSSAV  
RLRSSMPGVRLLQDSVD85FSLADAINTEFNTRTNEKVELQELNDRFANYIDKVRFLQQNKILL  
AELEQLKGQGSRLGDLYEEMRELRRQVDQLTNDKARVEVERDNLAEDIMRLREKLQEMLQREE  
AESTLQSFQDVDNASLARLDLKRKVESLQEEIAFLKKLHDEEIQELQAQIQEHVQIDVD259VS  
KPDLTAAALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEANRNNDALRQAKQESNEYRRQVQSLT  
CEVDALKGKTNESLERQMREMEENFALEAANYQDTIGRLQDEIQNMKEEMARHLREYQDLLNVKMAL  
DIEIATYRKLLGEESRISLPLPNFSSNLRETNLESPLVDTHSKRTLLIKTVETRDGQVINETSQHHD  
DLE

(b)

Spot 28



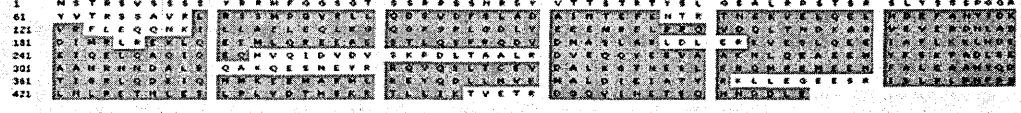
Spot 40



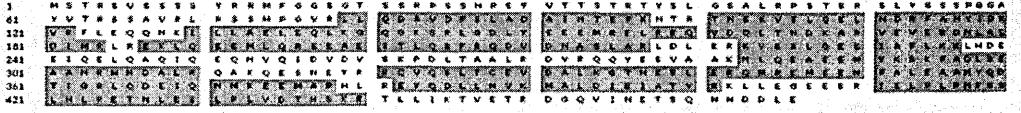
Spot 60



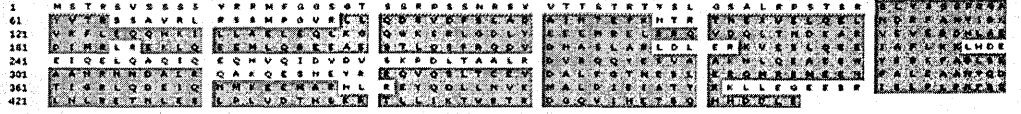
Spot 64



Spot 82



Spot 83



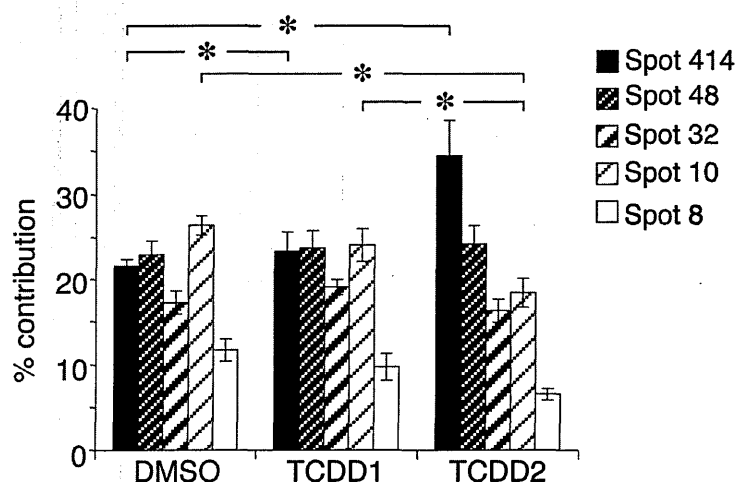
**Figure 25.** Panel (a) Rat vimentin aminoacid sequence with schematic representation of caspase-3 cleavage site (DSVD85/F) and caspase-8 cleavage site (IDVD259/V) as reported by Morishima et al. 1999. Panel (b) Graphic representation of the peptide coverage found for each vimentin spot identified by MS/MS. Dark areas indicate valid peptides used for protein identification. Spot numbers refer to **figure 23**.



#### 4.3.1.2. Proteins down-regulated following 10 days of TCDD exposure

As shown in **figure 24, panel B**, differentiating osteoblasts exposed to TCDD showed a significantly reduced expression of 13 protein species (9 individual proteins) in comparison to their untreated counterpart.

I identified 5 isoforms of lamin A (LMNA, spots # 8, 10, 32, 48, 414), whose expression levels were more than halved by TCDD at both doses. Lamin A is one of the major structural components of the nuclear envelope [173]. The presence of multiple isoforms with a similar molecular weight but a different pI, suggested the presence of post-translational modifications (PTM). The shift toward the acidic region of the 2-DE gel in the majority of these isoforms might indicate that PTM such as phosphorylation might have occurred. Indeed lamin A has multiple phosphorylation sites that control the assembly dynamics of lamins [174, 175]. Interestingly the most acidic isoform of lamin A (spot #414) made a significantly higher contribution (35%) to the total normalised volume of the sum of isoforms in TCDD-exposed osteoblasts than in non-exposed cells (25%) (**figure 26**).



**Figure 26.** Contribution of each lamin A isoform to the sum of the isoforms in osteoblasts exposed or not to two doses of TCDD (TCDD1: 1nM and TCDD2: 100nM). Each bar represents the average contribution of each lamin A INCA-normalised spot volume to the total INCA-normalised volume of the sum of lamin A spots (mean  $\pm$  SD). Asterisks indicate significant changes (one-way ANOVA, Tukey Kramer HSD  $p < 0.05$ ). Spot numbers refer to **figure 23**.

Both TCDD doses reduced the expression of alpha, beta and gamma subunits of the hetero-oligomeric T-complex protein 1 (CCT1, CCT2, CCT3, spot # 76, 294, 38 respectively), a molecular chaperone that assists protein folding upon ATP hydrolysis [176].

Similarly, a significant decrease was also observed in TCDD-exposed osteoblasts for (i) Ras-related protein RAB11A (RAB11A, spot #94), which governs endosomal trafficking, (ii) phosphoribosylaminoimidazolecarboxamide formyltransferase (ATIC, spot #35), a metabolic enzyme involved in the purine biosynthesis, and (iii) the 20S proteasome subunit alpha type 6 (PSMA6, spot #472).

The expression of annexin II (ANXA2, spot #90), a calcium regulated membrane binding-protein, appeared to be significantly reduced only by the high dose of TCDD.

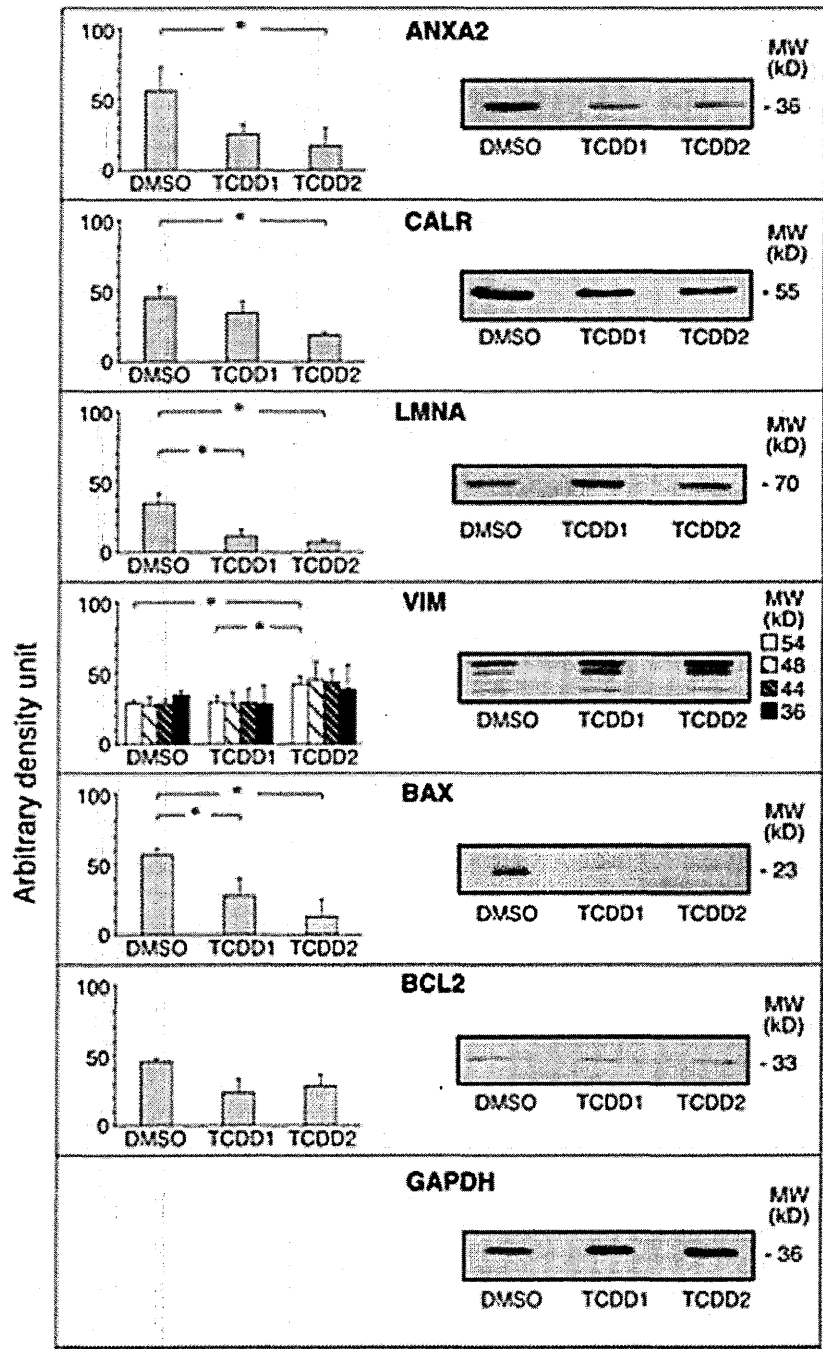
Notably, TCDD had a striking effect on a further calcium-binding protein, calregulin (CALR, spot #11). The expression of calregulin was 1.8- to 3.5 times lower in TCDD-exposed than in untreated osteoblasts. The difference between expected and experimental MW of calregulin observed on 2-DE gel (**table 5**) is not surprising, since the electrophoretic mobility of CALR is known to be anomalous, with the 46-kD protein showing 2-DE migration compatible with a molecular size of 60-kD [177].

#### 4.3.1.3. Western Blot Analysis

Changes in the expression of some proteins identified by MS/MS were confirmed by Western blotting. Three proteins whose expression was low in TCDD-treated osteoblasts were selected for analysis: the two calcium-binding proteins annexin A2 and calregulin and the structural component of the nuclear envelope lamin A. The direction of changes in expression was fully consistent with the proteomic data (**figure 27**).

I also confirmed the TCDD-induced increase in expression of vimentin. As shown in **figure 27**, osteoblasts exposed to TCDD had significantly higher expression of full length vimentin (54 kDa MW) than untreated cells, in accordance with my proteomic data. I also noted vimentin immunoreactive polypeptides at 48, 44 and 36 positions in both control and treated groups. A similar pattern of vimentin fragmentation has been previously associated with caspase-specific cleavage [167, 168]. However, the fragmentation profile in Western blot analysis, albeit in accordance with the literature, was not supported by my preliminary sequence analysis by tandem mass-spectrometry (see **figure 25**). I can offer no plausible explanation for this difference. To my knowledge, there are no reports of direct confirmation (*e.g.* protein sequencing by mass-spectrometry or Edmann degradation) of the precise size and sequences of the vimentin fragments derived from caspase-cleavage.

Western blotting of cell lysates from untreated and TCDD-exposed osteoblasts was also used to investigate some molecular components regulating apoptosis. A balance between the Bcl-2 and Bax members is essential to the cell's apoptotic potential, low apoptotic activity often being associated with low Bax/Bcl-2 expression ratio. As shown in **figure 27**, osteoblasts exposed to both TCDD doses showed much lower expression of the pro-apoptotic Bax protein than their untreated counterparts. The effect on Bcl-2 was less evident, though the Bax/Bcl-2 expression ratio was lower in the exposed osteoblasts.



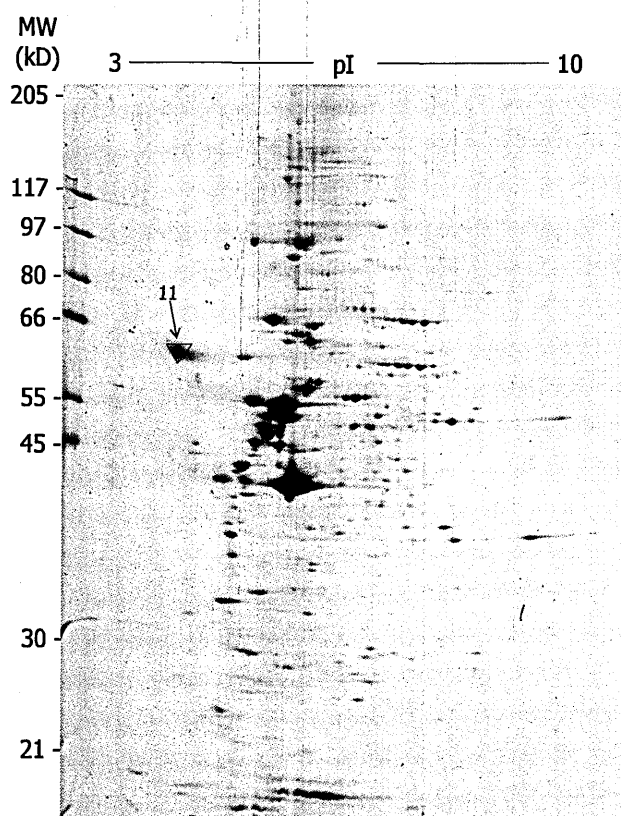
**Figure 27.** Changes in annexin A2, calregulin, lamin A, vimentin, BAX and BCL2 protein levels in differentiating osteoblasts untreated (DMSO) or after 10 days of exposure to 1nM TCDD (TCDD1) and 100nM TCDD (TCDD2) analyzed by Western blot. Protein bands in the autoradiograms were quantified by scanning densitometry. Expression data were normalized relative to GAPDH. The histograms show the band intensities from three independent experiments. Each bar represents the average normalized volume density  $\pm$  SD. The vertical axis shows arbitrary density unit. Asterisks mark significant expression changes (one-way ANOVA, Tukey Kramer HSD  $p < 0.05$ ).

#### ***4.3.2. Proteome profile of differentiating osteoblasts exposed to TCDD at earlier time points.***

I have previously reported (section 4.3.1) that in differentiating osteoblasts, 27 protein species, corresponding to 18 individual proteins, showed a statistically significant change in abundance after 10 days of TCDD exposure, mainly at the highest dose (100nM). I was therefore interested in investigating the expression profile of these 27 species at earlier times of TCDD exposure (3 and 7 days), where no effect on the expression of osteogenic markers was found.

##### ***4.3.2.1. Proteome profile of differentiating osteoblasts exposed for 7 days to TCDD.***

The protein expression pattern of differentiating osteoblasts exposed to 100nM TCDD for 7 days was compared with their untreated counterpart using 2-DE image analysis software. **Figure 28** shows the 2-DE average gel representative of unexposed osteoblasts after 7 days of culture



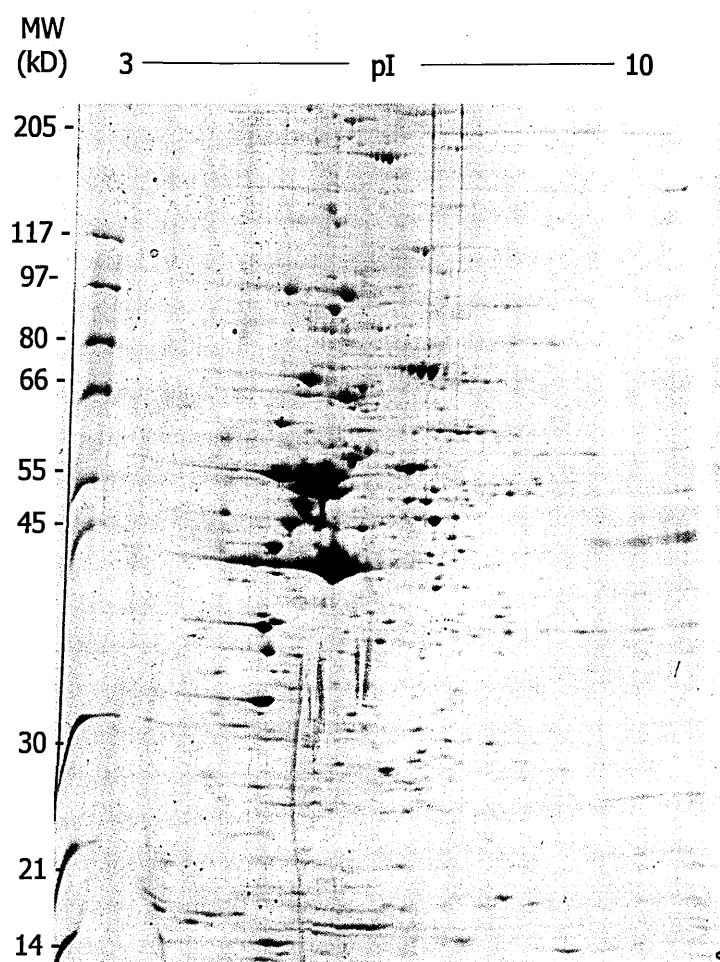
**Figure 28.** Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate (200 µg proteins) from unexposed osteoblasts (DMSO). The average gel generated by image analysis of four replicates samples is shown. The protein spot, whose abundance is significant decreased by 7-days of TCDD exposure is highlighted by the triangle.

As shown in **figure 28**, only one out of the selected 27 protein species was significantly altered in its expression level: the abundance of calregulin (spot #11,  $p < 0.01$ ) was approximately halved by TCDD treatment.

#### 4.3.2.2. Proteome profile of differentiating osteoblasts exposed for 3 days to TCDD

The protein expression pattern of differentiating osteoblasts exposed to 100nM TCDD for 3 days was compared with their untreated counterpart using 2-DE image analysis software. **Figure 29** shows the 2-DE average gel representative of unexposed osteoblasts after 3 days of culture.

None of 27 monitored protein species showed any statistically significant change in the expression level after 3 days of TCDD exposure.



**Figure 29.** Colloidal Coomassie Brilliant Blue-stained 2-DE gel of whole cell lysate (200 µg proteins) from unexposed osteoblasts (DMSO) at three days of cell culture. The average gel generated by image analysis of four replicates samples is shown.

3-days of TCDD exposure did not affected significantly the abundance of any protein spots.

No further protein out of the monitored 27 protein species showed any change in abundance unique to 3 and/or 7 days of exposure.

#### ***4.3.3. Time-course analysis of the identified proteins susceptible to TCDD in relation to the progress of osteoblastic differentiation***

I explored the expression profile of the identified protein species susceptible to TCDD effect, in relation to the progress of osteoblastic differentiation, comparing their expression in untreated differentiating osteoblasts at 3, 7, and 10 days of culture in osteogenic conditions.

As shown in **figure 30**, the majority of the identified TCDD susceptible proteins showed significant expression changes as osteoblasts differentiated.

On the basis of these changes, proteins might be grouped into 3 main classes.

*The first class* includes proteins, whose expression gradually and significantly increased with the progress of differentiation. Most striking was the increase in the expression of calregulin (spot#11) after 10 days, compared to days 3 and 7 (9-fold,  $p < 0.001$  and 5.5-fold  $p < 0.01$  respectively) (**figure 30, panel A**).

A similar pattern of expression was observed for all the lamin species, whose levels were higher at day 10 (spot #8, 10, 32, 48, 414, range 1.5-3-fold,  $p < 0.001$ ) than at day 3 of culture. Interestingly, also the expression of annexin A2 was significantly increased at day 10 relative to day3 (3-fold,  $p < 0.01$ ). At day 10, the expression of two vimentin species (spot #60, #82) was between 2 and 3 times higher than at early time points ( $p < 0.01$ ). Moreover this trend was also observed for other vimentin species (#28, #40) although it did not reach statistical significance.

Ten days of culture significantly increased the levels of RAB11A (spot #94, 4.4-fold,  $p < 0.01$ ), ATIC (spot #35, 3.1-fold,  $p < 0.001$ ), and cytosolic chaperonins CCT1, CCT3

(spot #76 and #38, 2.1-3.3-fold respectively,  $p < 0.001$ ), but had a less pronounced effect on chaperonin CCT2, whose difference in abundance did not reach statistical significance.

*The second group* of proteins includes those, whose expression gradually decreased as cells differentiated. TPM1, TPM2 and TPM4 exhibited a significantly reduced expression at day 10, compared with day 3 (spots #66, #69, #80, 2.3-, 2.4- and 1.88-fold respectively,  $p < 0.001$ ). This trend was also found for one vimentin isoform (spot #83) whose abundance was 1.7-fold lower at 10 than at 3 days ( $p < 0.01$ ).

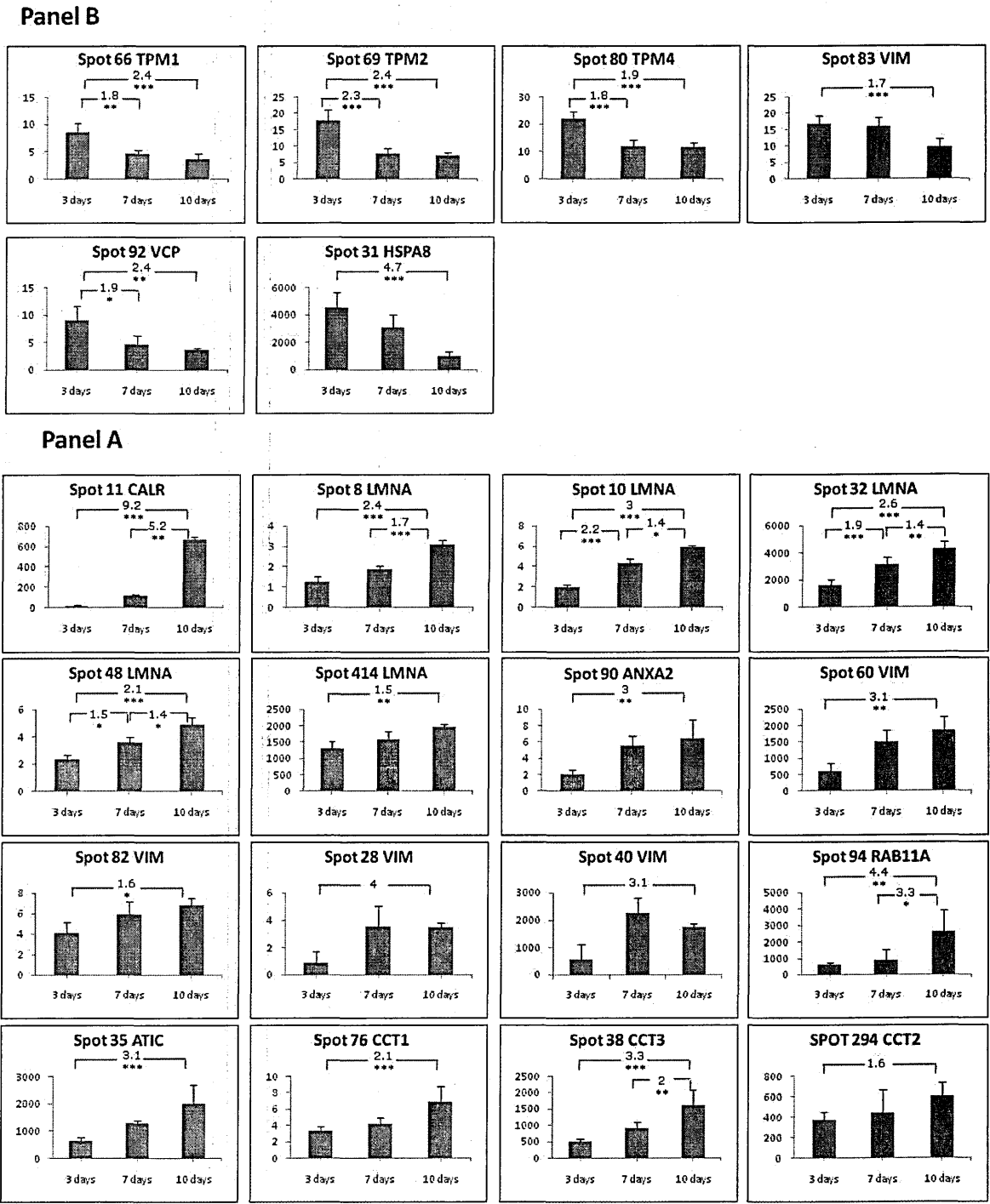
A more pronounced decrease in abundance at the differentiation stage was also observed for VCP (spot #92, 2.44-fold,  $p < 0.01$ ) and HSPA8 (spot #31, 4.75-fold,  $p < 0.001$ ) (**figure 30, panel B**).

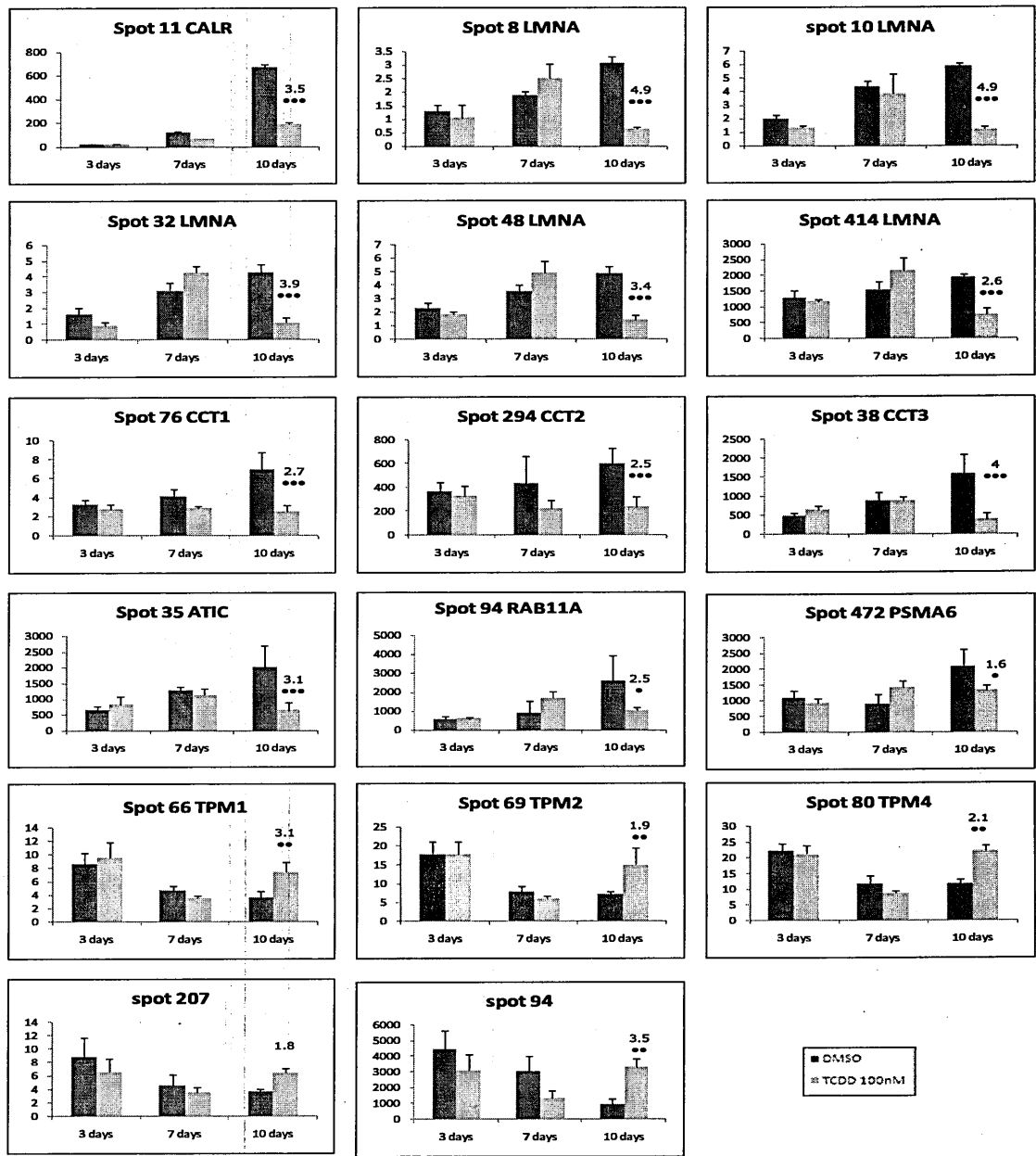
*The third group* includes few whose expression levels did not change significantly in relation to the length of culture.

Notably, many of the observed patterns of expression accompanying the differentiation progress were reversed by 10 days of TCDD exposure as shown in **figure 31**.

This was the case for calregulin and all the lamin A species that, while increasing their abundance during differentiation, were down-regulated by 10 days of exposure to TCDD. The same was true for CCT1, CCT2 and CCT3, ATIC, RAB11A and PSMA6. Interestingly, all proteins within the second group (TPM isoforms, VCP and HSPA8), whose reduced expression was observed as osteoblast differentiated, were up-regulated after 10 days of TCDD exposure.







**Figure 31.** Expression levels for the selected proteins whose patterns of expression accompanying the differentiation progress were reverted by 10 days of TCDD exposure. Each bar represents the average spot abundance expressed as INCA normalised volume  $\pm$  SD. The vertical axis shows the INCA normalised spot volume. Numbers indicate the difference in protein expression (-fold). Dots highlight significant expression changes by TCDD treatment (one-way ANOVA, Tukey Kramer HSD. Three dots indicate  $p < 0.001$ , two dots indicate  $p < 0.01$ , one dot indicates  $p < 0.05$ ). Spot numbers refer to **figure 23**.

#### **4.3.4. Differential proteomic analysis using univariate approach: remarks**

Three major conclusions can be drawn from these proteomic results.

*First*, I have shown that at early time points of TCDD exposure, when cells are still proliferating (3 days) or/and enter in the differentiation stage (7 days), TCDD had no significant effect on proteome profile. The only exception is the significantly decreased expression of calregulin occurring after 7 days of TCDD treatment. Calregulin is a chaperone protein that regulates  $\text{Ca}^{2+}$  levels by modulation of the endoplasmatic reticulum (ER)  $\text{Ca}^{2+}$  storage and transport [178]. Thus, the reduction of calregulin might represent an early marker of the TCDD-induced disruption of calcium homeostasis, since calregulin is also down regulated after 10 days of exposure, at which time calcium deposition is significantly decreased in TCDD-exposed osteoblasts (section 4.1.2).

*Secondly*, after 10 days of TCDD exposure, at which point the osteoblasts should be fully differentiated, the proteome profile has been significantly altered.

TCDD strongly induced the expression of *cytoskeletal components* such as TPM, vimentin and ACTG1. All these proteins are implicated in one or more steps of cytoskeleton protection, stress fiber assembly and cell motility [162, 163, 179-181].

Interestingly TCCD down-regulated the expression of two important *calcium-binding proteins*: calregulin and annexin A2. Calregulin is a multifunctional protein that participates in calcium homeostasis via its high calcium storage capacity [177, 178, 182]. Annexin A2 serves as a  $\text{Ca}^{2+}$  channel in the plasma membrane and in matrix vesicles, which have the vital role of initiating mineralization in bone [183]. Under-expression of these proteins might therefore interfere with calcium transport and deposition, and indeed it was significantly decreased after 10 days of exposure to TCDD (section 4.1.2).

Further important pathways influenced by TCDD are related to *protein quality control and trafficking*. In TCDD-exposed osteoblasts there was a significant decrease in the expression of chaperonins containing T-complex polypeptides 1, 2 and 3 (CCT1, CCT2, CCT3) which assist in the folding of actin and tubulin, suggesting dysregulation of protein-folding and possible abnormalities in cytoskeleton organization.

The intracellular trafficking machinery of osteoblasts is also likely to be impaired by the down-regulation of RAB11A, a member of the large superfamily of small Ras-related GTP-binding Rab proteins, acting as master signal components that regulate many aspects of vesicular transport along recycling, endocytic and exocytic pathways. In this scenario, the TCDD-enhanced expression of PSMC2, a 26S subunit regulatory unit, and of PSMB4, the 20S catalytic/enzymatic core, together with the down-regulation of PSMA6, the alpha-type subunit of the 20S proteasome, although apparently contradictory, might be simply a sign of changes in the proteasome subunit composition and activity after exposure to TCDD.

The TCDD-induced up-regulation of the chaperonin HSPA8 may be an adaptive response to reinforce the protein-folding control process and to boost tolerance to stressful conditions [184].

*Thirdly*, I found that TCDD exposure *counteracts* the expression profile of proteins associated with the duration of culture in osteogenic conditions, *i.e.* associated with normal growth and differentiation kinetics.

Calregulin was strongly up-regulated during osteoblast differentiation, increasing 9-fold at 10 days of culture compared to three days. This trend is consistent with the reported increase in calregulin expression accompanying the differentiation of osteoprogenitor cells in mouse bone marrow into preosteoblast cells upon parathyroid hormone treatment [185]. Thus, calregulin may participate in an intracellular  $\text{Ca}^{2+}$  transport pathway and could function as a  $\text{Ca}^{2+}$  carrier to the mineralization front. Such

role would be dramatically *jeopardized* by TCDD, which might impair the osteoblast mineralization process, reducing the level of calregulin.

TCDD also reversed the expression trends of annexin A2, lamin A, RAB11A and chaperonins CCT1, CCT2, CCT3. These proteins showed a gradual up-regulation during differentiation in accordance with their functional role in growth and differentiation. In fact the expression trend of annexin A2 and RAB11A is consistent with their role in the progression of mineralization [183] and of secretory functions in differentiated osteoblasts [186]. Enhanced expression of lamin A is considered an early marker of differentiation, and it is speculated that the mechanism may involve increased nuclear rigidity [187]. Cytosolic chaperonins CCTs have been shown to play an important role in cell growth and differentiation by assisting in the folding of tubulin and other proteins [176].

The drop of the expression levels of all these proteins caused by TCDD may thus impair osteogenic progression and the functionality of osteoblasts by lowering their secretory capacity, ability to maintain the differentiated state, protein folding efficiency and control of trafficking.

Cellular differentiation, associated with phenotypic modifications of the cell, includes reorganization of the cytoskeleton involving changes in cell shape and cell contacts. During osteogenic differentiation, MSCs change from their characteristic fibroblast-like phenotype to a near spherical shape characteristic of the differentiated stage, with a consequent change in the kinetics of dynamic assembly and disassembly of microfilaments [188].

In my model, osteoblast differentiation was accompanied by a gradual decrease in the expression of tropomyosin, a major microfilament stabilizing component, consistent with the tropomyosin reduction reported in differentiating systems of non-muscle cells [189, 190]. In parallel I observed a differential expression of type III intermediate filament vimentin spot83, which was reduced after 10 days of culture, when the

majority of cells have changed their shape to the more rounded form associated with the differentiated state [185, 190].

Such expected changes in cytoskeletal organization during the osteogenic differentiation were again reversed by 10 days of TCDD exposure, which causes enhanced expression of all TPM isoforms and vimentin spot 83.

#### **4.4. Differential proteomic analysis using a multivariate approach for data assessment**

Quantitative 2-DE gel data typically comprise many variables (protein species) but few observations (experimental replications). Multivariate analyses have been proposed to aid in the evaluation of proteomics experiments, since in general they cope well with data sets where the number of observations is much smaller than the number of variables [191]. Instead of analyzing each protein variable on its own (as in the univariate approach), all protein variables can be evaluated at the same time using multivariate techniques to look for patterns in expression changes. The type of multivariate analysis already applied to proteomic data is a method for dimension reduction known as principal component analysis (PCA) [192]. PCA is an unsupervised technique (*i.e.* it does not use any knowledge of the groupings of the data) which provides a simplified graphical representation of the multidimensional data.

Therefore I have applied PCA to my whole study as an exploratory data analysis method able to visualize differences between complex proteome profiles.

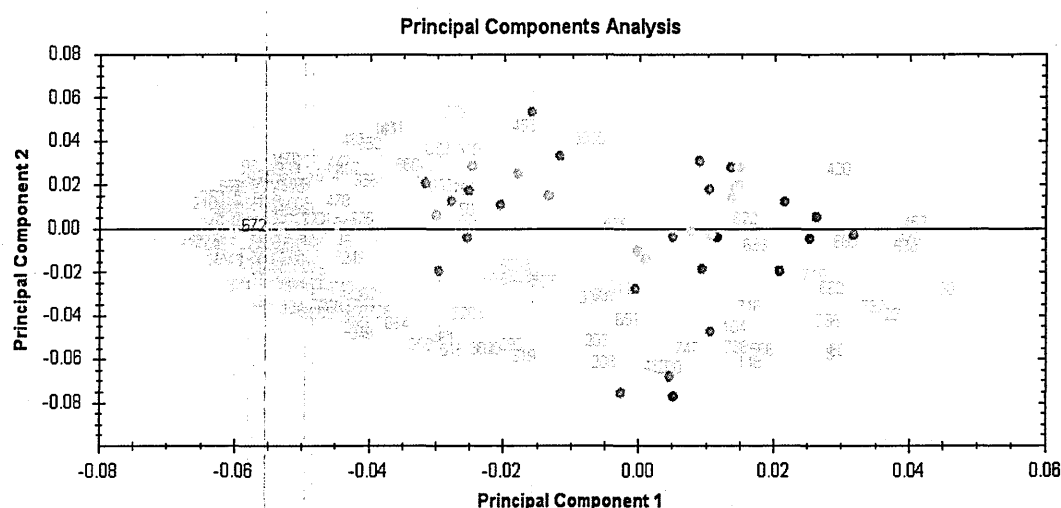
I used new software that has been recently obtained by my laboratory, the *Progenesis SameSpots and Stats* package (Nonlinear Dynamics, UK).

The unique approach of *Progenesis SameSpots and Stats* produces results with no missing values, so both univariate and multivariate statistical techniques can be applied for a complete exploration of the protein expression data. The statistics package includes PCA and also Correlation Analysis and Hierarchical Clustering based on expression profile data. Furthermore the software provides the q-value for each tested spots. The q-value controls the false discovery rate (FDR) where the focus is on achieving an acceptable ratio of true and false positives. For example, a q-value of

0.05 (FDR = 5%) means that on average 5% of changes identified as significant would be expected to have arisen from type I error (a protein species is declared to be differently expressed erroneously). In this study, the level of significance has been set up at  $FDR < 5\%$ .

The starting point was to apply PCA with a low level of assumption, which means including in the analysis all the spots automatically identified as interesting in accordance to ANOVA p-values ( $p < 0.05$ ) when all treatments groups are included (unexposed and 2-doses TCDD exposed groups at 3, 7 and 10 days of exposure).

The visualisation of samples (group treatment) and features score (protein spots score) and as loading plots on PCA graph is shown in **figure 32**.



**Figure 32.** PCA score and loadings plot of the two first PCs of the whole dataset. PC1: 46.27%, PC2: 17.34%. Pink, light blue and purple dots refer to 2-DE gels from 3-days exposure, where pink highlights untreated samples, light blue TCDD 1nM treated samples, purple TCDD 100 nM treated samples. Light orange, pale blue and orange dots refer to 2-DE gels from 7-days exposure, where light orange highlights untreated samples, pale blue TCDD 1nM treated samples, orange TCDD 100 nM treated samples. Green, dark green and brilliant green dots refer to 2-DE gels from 10-days exposure, where green highlights untreated samples, dark green TCDD 1nM treated samples, brilliant green TCDD 100 nM treated samples. Numbers on the plot refer to discriminating spots number.

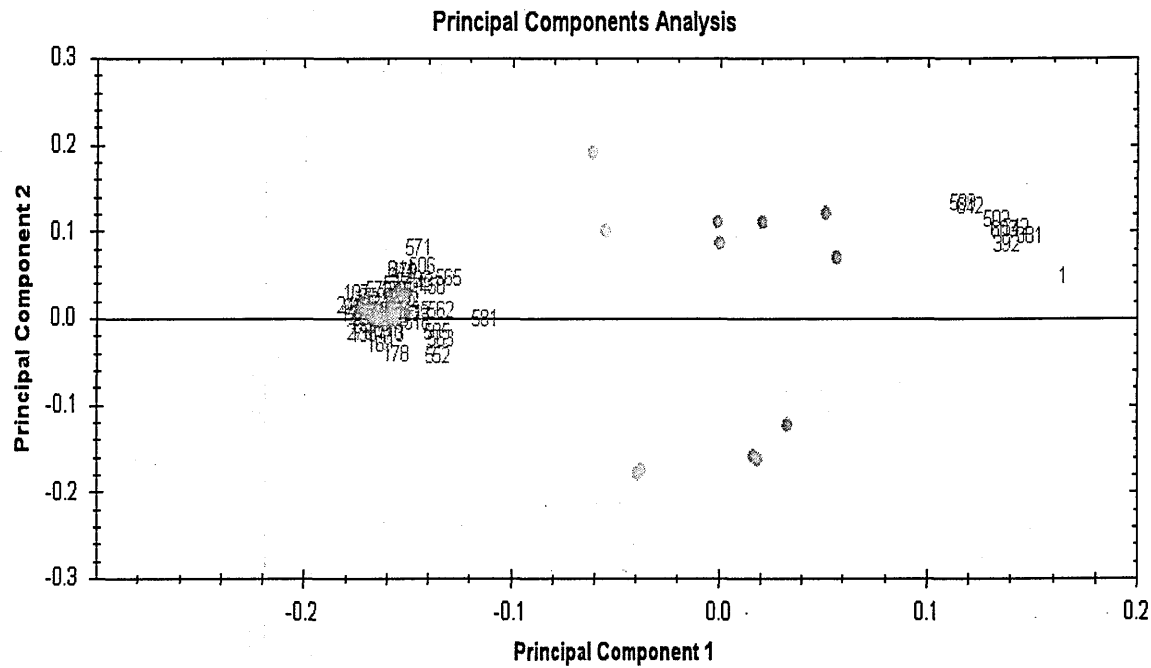
The first principal component (PC1) alone accounted for approximately 47% of the total variance contained in the original data set. It was able to separate two classes of



samples: <0, 3 days as whole; >0, 7 and 10 days as a whole, suggesting that the data showed a duration effect (culture days) rather than an exposure effect (TCDD doses) and that the variance increases over time.

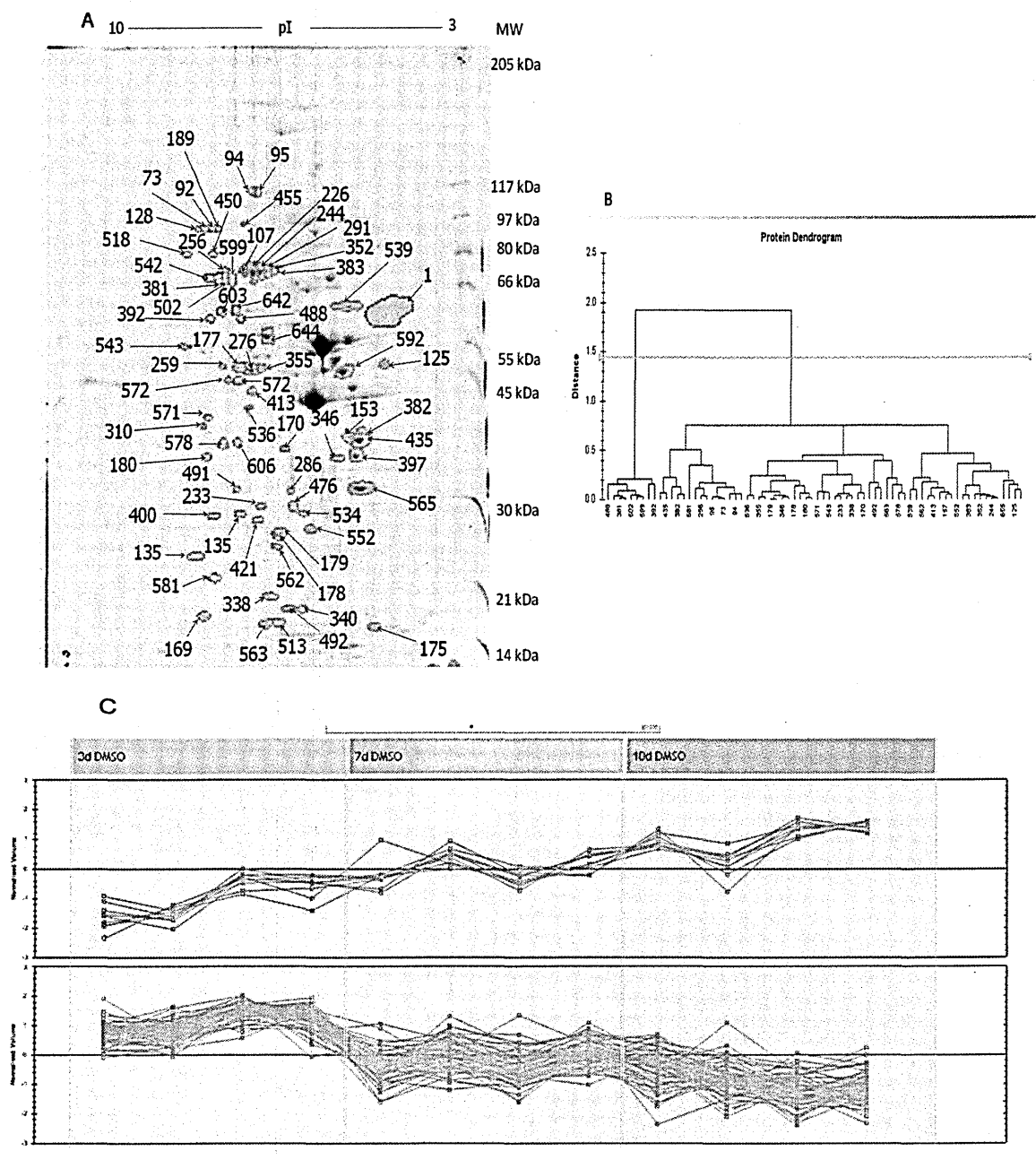
This preliminary evaluation prompted me to look exclusively within time differences (untreated osteoblasts culture days), excluding 2-DE gel images with TCDD doses from the PCA analysis,

As shown in **figure 33**, PC1 splits the data into two main groups: <0= untreated osteoblasts after 3 days of culture and >0= untreated osteoblasts after 7 and 10 days of culture, explaining 74% of the total variance.



**Figure 33.** PCA score and loadings plot of the two first PCs of the dataset referring to untreated samples. PC1: 74.95 %, PC2: 5.85 %. Pink dots refer to 2-DE gels from 3-days untreated samples, light blue to 7-days untreated samples, purple to 10-days untreated samples. Numbers on the plot refer to discriminating spots number.

This result was further investigated to evaluate the discriminating spots responsible for the clustering. These results are display in **figure 34**.



**Figure 34.** **Panel A:** discriminating spots location of 2-DE gel image. Red spot: proteins showing up-regulated expression with increasing length of culture time; blue spots: proteins showing down-regulated expression with increasing length of culture time. See **table 6** for protein identification. **Panel B:** dendrogram tree where similar protein expression profiles cluster together. **Panel C:** the corresponding expression profiles, where red line refers to proteins showing up-regulated expression with increasing length of culture time, blue line to proteins showing down-regulated expression with increasing length of culture time.

In **figure 34**, the 69 discriminating spots ( $FDR < 5\%$ ) are highlighted on the gel image together with their pattern of expression. There were 8 protein species whose expression was up-regulated and 61 whose expression was down-regulated with increased culture duration.

The 74% of these spots (51 out of 69) identified by MS/MS are reported in **table 6**. The identified protein species corresponded to 34 individual proteins, whose functional classification is reported in *section 4.6*.

Among these 69 spots, there were 6 protein species that lay further away from the plot origin, suggesting that they had the strongest impact on the model.

In fact, when the PCA analysis was restricted to these features, PC1 explained 93% of the variance (**figure 35**) and the power of the analysis was more than 80% (data not shown). These protein features were grouped by their expression patterns using correlation analysis and hierarchical clustering. One protein species, namely calreticulin, was up-regulated during the process of osteoblast differentiation, rising above the 3 days level at 10 days of culture. The remaining five (NPM1, HSPb1 and 3 isoforms of CALD) were all reduced in abundance after 7 days of culture and reached a minimum at 10 days.

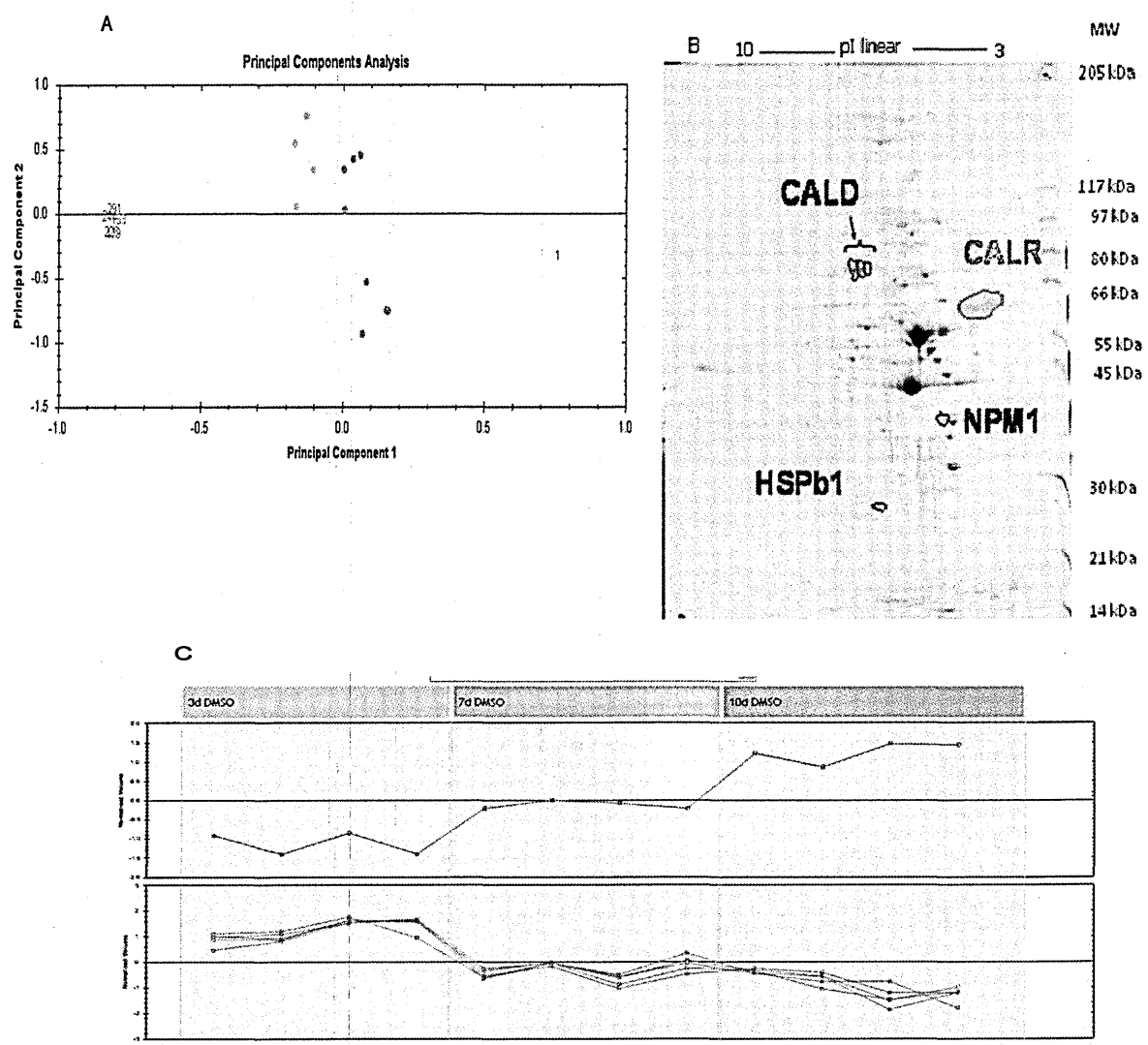
**Table 6.** Identification of proteins showing different levels of expression characterizing untreated osteoblast proteome profile at different time points, by 2 different LC-MS/MS platforms. Detailed information on peptide/protein identification is reported in **supplemental table 2** (Appendix 7.3).

Spot #	Identified protein	Symbol	Uniprot-Swiss-Prot #	pI Theor./Exp. <sup>a)</sup>	MW (kD) Theor./Exp. <sup>a)</sup>	LC-ESI-IT			LC-ESI-Q-TOF		
						# unique pept. <sup>b)</sup>	Cov% <sup>c)</sup>	Score <sup>d)</sup>	# unique pept. <sup>b)</sup>	Cov% <sup>c)</sup>	Score <sup>d)</sup>
1	Calregulin	CALR	P18418	4.3/4.3	48/58	16	51%	136.6	5	20%	45.4
73	Elongation factor 2	EEF2	P05197	6.5/7.2	95.1/96	5	8%	51.2	4	6%	32.0
92	Elongation factor 2	EEF2	P05197	6.5/7	95.1/96	2	4%	19.9			
95	Vinculin	VCL	gi149031251 <sup>e)</sup>	5.9/6.1	117/110	49	48%	478.6	4	4%	33.3
125	Vimentin	VIM	P31000	5.1/4.5	53.7/45	62	72%	659.3			
128	Elongation factor 2	EEF2	P05197	6.5/7.3	95.1/96				5	9%	48.1
135	Phosphoglycerate mutase 1	PGAM1	P25113	7.3/7.9	28.7/30	11	42%	102.7	9	51%	94.9
153	nucleophosmin 1	NPM1	P13084	4.6/5.8	32.6/35	12	35%	109.3	1	8%	9.1
169	Nucleoside diphosphate kinase B	NME2	P19804	7.8/7.8	17.3/16	6	40%	52	10	63%	85.9
170	60S acidic ribosomal protein	RPLP0	P19945	6.1/6	34.2/35	14	35%	144.7	9	47%	98.8
177	Alpha-enolase	ENO1	P04764	6.4/6.7	47.1/48	25	54%	235.6			
179	Heat shock protein beta-1	HSPB1	P42930	6.4/6.4	22.9/24	10	50%	104.2	9	52%	87.3
180	PDZ and LIM domain protein 1	PDLIM1	P52944	7.1/7.2	35.6/37	3	11%	28.1	2	9%	18.3
226	L-caldesmon	CALD	Q62736	6.6/6.7	60.6/66	34	47%	366.1	9	16%	76.8
233	Proteasome subunit beta type 7	PSMB7	Q9JHW0	8.4/6.8	30/30				4	23%	43.4
244	L-caldesmon	CALD	Q62736	6.6/6.5	60.6/66	24	37%	268.4	10	19%	83.4
276	Alpha-enolase	ENO1	P04764	6.4/6.6	47.1/48	28	55%	306.8			
291	L-caldesmon	CALD	Q62736	6.6/6.4	60.6/66	16	30%	178.5			
338	ADP-ribosylation factor 1	ARF1	P84079	6.8/6.4	20.7/21				7	48%	65.6

346	Emerin.	EMD	Q63190	5.1/5.1	29.7/36	6	30%	61.5				
352	L-caldesmon	CALD	Q62736	6.6/6.3	60.6/66	13	26%	141.2	2	5%		13.8
355	Alpha-enolase	ENO1	P04764	6.4/6.4	47.1/48	18	48%	172.2				
381	Lamin-A.	LMNA	P48679	6.7/7.2	74.3/66	48	50%	446.7	7	12%		59.3
382	Tropomyosin alpha-1 chain	TPM1	P04692	4.8/5	32.7/36	9	25%	101.5	8	36%		81.2
392	Bifunctional purine biosynthesis protein PURH (ATIC)	ATIC	O35567	6.9/7.4	64.2/64	7	19%	68.8				
397	Tropomyosin alpha-1 chain	TPM1	P04692	4.8/5	32.7/34	32	64%	311.5	12	43%		125.1
400	Phosphoglycerate mutase 1	PGAM1	P25113	7.3/7.2	28.7/29	9	42%	91.8	11	45%		110
413	Ornithine aminotransferase	OAT	P04182	6.8/6.7	48.3/47	19	36%	189.3	13	35%		151
421	Endoplasmatic reticulum protein 29	ERP29	P52555	6.8/7	28.6/29	11	38%	94.8	6	24%		50.8
435	Tropomyosin beta	TPM2	P58775	4.7/4.8	33/36	37	82%	371.4	11	40%		120.8
455	Programmed cell death 6-interacting protein	PDCD6IP	Q9QZA2	8.8	44/80	5/7	13%	48.2	6	19%		55
476	Proteasome activator complex subunit 1	PSME1	Q63797	6.0	28.6/30	6/5.9	23%	48.9				
479	Lamin-A	LMNA	P48679	6.7/6.7	74.3/66	9	17%	90				
502	Lamin-A	LMNA	P48679	6.7/7.1	74.3/66	6	11%	69.2	4	22%		39.8
518	Aconitate hydratase	ACO2	Q9ER34	7.4/7.4	82.5/80				3	6%		31.6
536	Septin-2	SEPT2	Q91Y81	6.3/6.4	41.6/43	12	39%	116	7	27%		66.9
539	Beta-actin	ACTB	P60711	5.4/5	41.6/55	2	10%	24.5	10	23%		105.1
542	Lamin-A.	LMNA	P48679	6.7/7.4	74.3/66	25	42%	269.5				
543	Adenylyl cyclase-associated protein 1	CAP1	Q08163	7.8/7.8	51.5/53				14	39%		133
552	Heat shock protein beta-1	HSPB1	P42930	6.4/5.8	22.9/24	6	23%	51.4	7	33%		68.2
562	Proteasome subunit beta type 4	PSMB4	P34067	6.9/6.2	29.2/26	6	26%	58.4	5	22%		53.6
565	Tropomyosin alpha-4	TPM4	P09495	4.7/4.8	28.5/32	29	73%	287.2	10	57%		98.6
572	Elongation factor 1-gamma	EEF1G	Q68FR6	6.5/6.8	50/48	6	15%	59.2	8	20%		81.3

578	LIM and SH3 domain protein 1	LASP1	Q99MZ8	7.0	30/34	4/6.5	19%	44.9	2	10%	18.8
585	GTP-binding nuclear protein Ran	RAN	P62828	7.9/8	24.3/24	6	33%	57.5	5	31%	47.8
592	Vimentin	VIM	P31000	5.1/5	53.8/47	62	72%	659.3	15	36%	159.6
599	Lamin-A	LMNA	P48679	6.7/7	74.3/66	16	28%	163.5			
603	Lamin-A	LMNA	P48679	6.7/7.4	74.3/66	48	50%	446.7	8	12%	73
606	Annexin A2	ANXA2	Q07936	8.1/7.2	38.5/38	6	23%	57.4			
642	Lamin-A	LMNA	P48679	6.7/7	74.3/60	35	44%	312.1	9	13%	80.7
644	Protein disulfide-isomerase A3	PDIA3	P11598	6.0/6.2	56.6/57	39	57%	366.9	17	41%	162

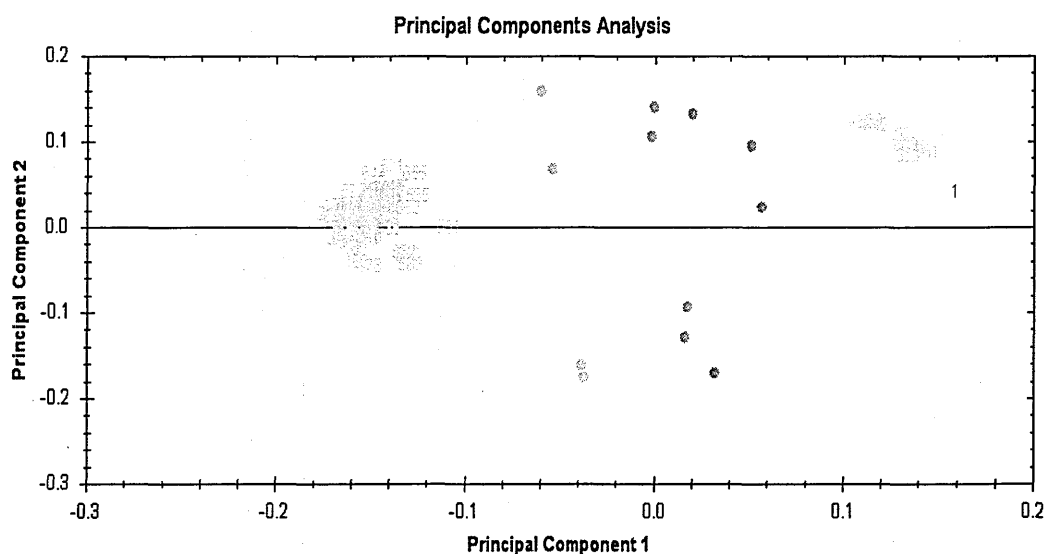
- a) Theor.: theoretical, data-based annotations; Exp.: experimental, from 2-D gels.
- b) # unique pept.: number of unique peptide matches found for the given protein.
- c) Cov%: all amino acids from valid peptide matches as a percentage of the total number of amino acids in the protein.
- d) Score: the protein score is calculated from the individual normalized z-scores of validated peptides. Peptide z-score refers to the distribution of calculated scores compared to that of random peptide sequences in order to find the mean and variance ([www.phenyx-ms.com](http://www.phenyx-ms.com)).
- Database redundancy is handled and solved by the Phenyx software. If a protein shares all of its validated peptides with another protein, then it is considered to be a subset and will not appear in the best-scoring protein list. It appears in the protein Details panel under Subset for the principal and better scoring parameters. Therefore, the entries reported refer exclusively to the best-scoring protein found by the search engine.
- e) Rat vinculin is recognized only using NCBI nr database and not Uniprot- SwissProt. Therefore the Accession number of rat vinculin is reported as gi number.



**Figure 35. Panel A:** PCA score and loadings plot of the two first PCs of the dataset referring to untreated samples. PC1: 93%, PC2: 6%. Pink dots refer to 2-DE gels from 3-days untreated samples, light blue to 7-days untreated samples, purple to 10-days untreated samples. Numbers on the plot refer to discriminating spots number. **Panel B:** discriminating spots location of 2-DE gel image. Red spot: proteins showing up-regulated expression with increasing length of culture; blue spots: proteins showing down-regulated expression with increasing length of culture. **Panel C:** similar protein expression profiles are clustered together using correlation analysis and hierarchical clustering. Red line refers to proteins showing up-regulated expression with increasing length of culture, blue line to proteins showing down-regulated expression with increasing length of culture. See Appendix 7.2 for full protein names.

To explore further, I examined exposure differences within each time point, in case the cross time point effects were masking the treatment effects.

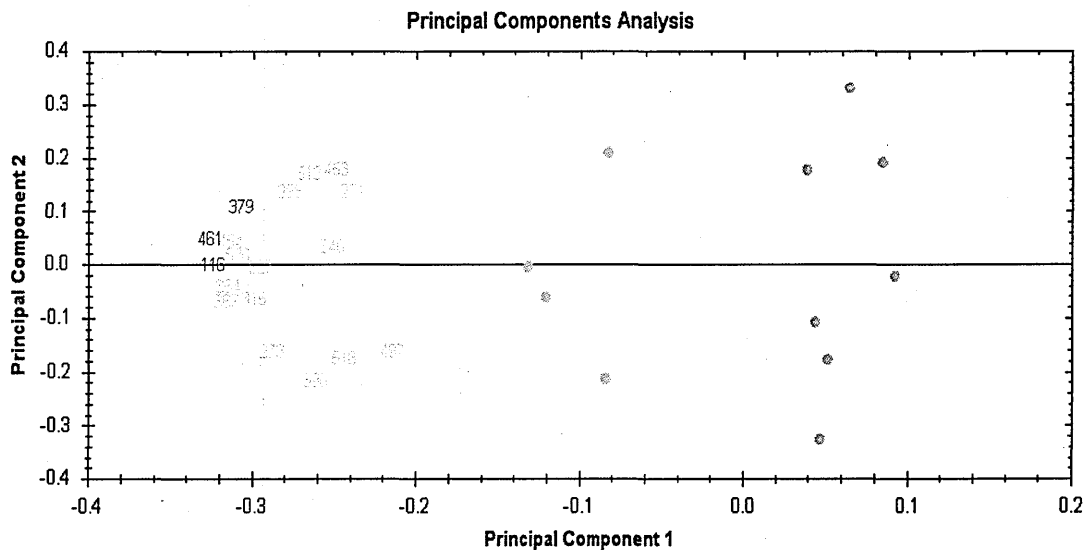
As shown in **figure 36**, TCDD exposure for 3 days had a relatively slight effect on the proteome profile, with PC1 explaining only 43% of the variance.



**FIGURE 36.** PCA score and loadings plot of the two first PCs of the dataset referring to 3-days untreated and TCDD treated samples. PC1: 43.16%, PC2: 33.05%. Pink dots highlight 2-DE gels from 3-days untreated samples, light blue from 3-days TCDD 1nM treated samples, purple from 3-days TCDD 100 nM treated samples. Numbers on the plot refer to discriminating spots number.

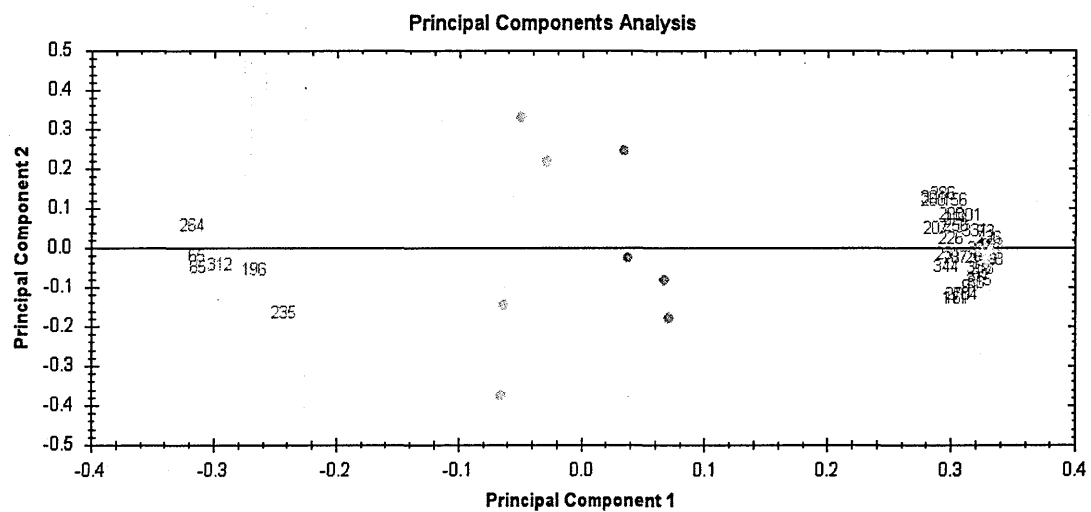
A more obvious effect of TCDD on osteoblast proteins was observed at 7 days of exposure, but only when the highest dose (100 nM) was considered (**figure 37**). PC1 explained 68% of the variance and the untreated samples were much better separated from TCDD treated samples.





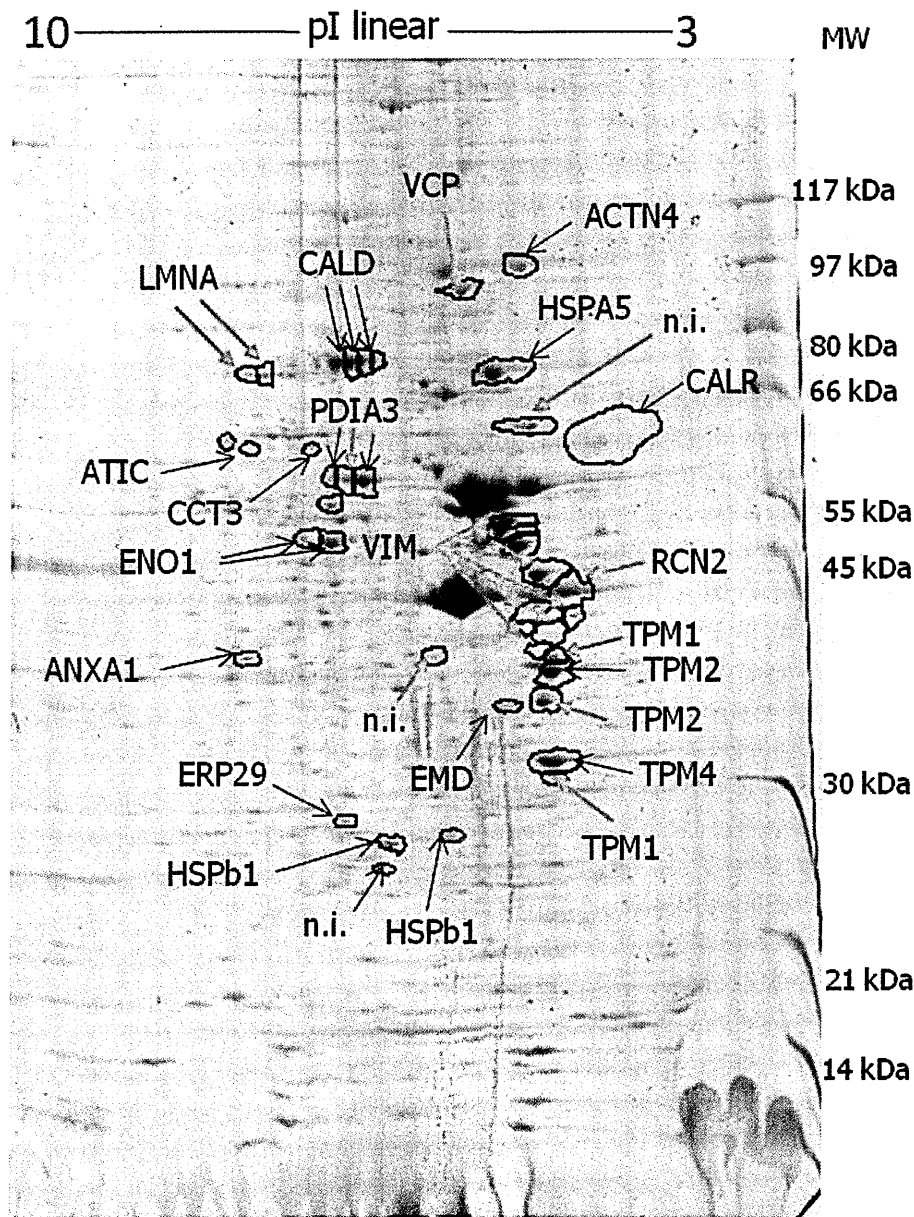
**Figure 37.** PCA score and loadings plot of the two first PCs of the dataset referring to 7-days untreated and TCDD treated samples. PC1: 68%, PC2: 12%. Pink dots highlights 2-DE gels from 7-days untreated samples, light blue from 7-days TCDD 100nM-treated samples. Numbers on the plot refer to discriminating spots number.

Similarly, PCA analysis showed a clearer effect of TCDD treatment when the exposure duration was 10 days (**figure 38**). At this time point, PC1 explained 85% of the variability in the dataset, when the control group was compared with the one exposed to the highest TCDD dose.



**Figure 38.** PCA score and loadings plot of the two first PCs of the dataset referring to 10-days untreated and TCDD treated samples. PC1: 85.79%, PC2: 4.87%. Pink dots highlights 2-DE gels from 10-days untreated samples, purple from 10-days TCDD 100 nM treated samples.

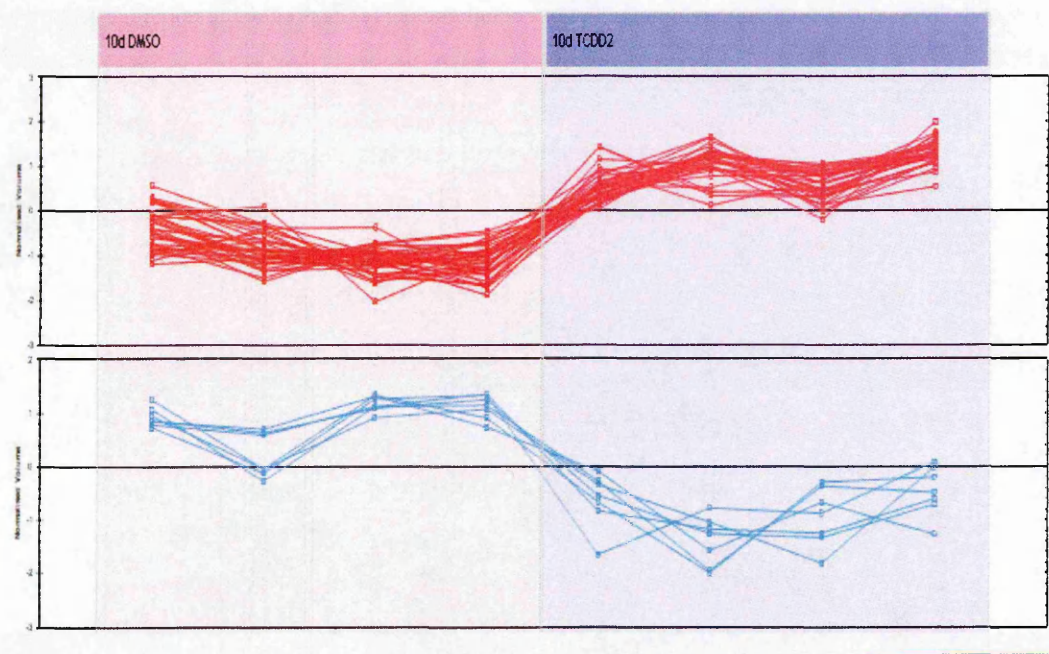
There were 42 discriminating protein species, out of them 19 had an FDR<5%. These spots are shown in **figure 39**. Mass spectrometric identification was successful for 38 spots (90%), corresponding to 19 individual proteins.



**Figure 39.** Location on the 2-DE gel image of the 42 discriminating spots for the PCA analysis of 10-days exposure untreated *vs* TCDD 100nM treated samples. Pink arrows highlight the protein species with FDR<5%. n.i. refers to unidentified proteins. See Appendix 7.2 for full protein names.

The expression cluster of the 42 spots is shown in **figure 40**. There were 8 protein species down regulated, whereas the remaining 38 were up-regulated by the highest dose of TCDD. The most significantly down-regulated proteins, (FDR<5%), were 2 isoforms of lamin A. Moreover two ATIC isoforms, CCT3, calregulin and one unidentified spot were down-regulated. In the up-regulated set of proteins there were 17 with FDR<5% (two unidentified identified spots, VCP, one PDIA3 isoform, RCN2, tropomyosin 1 and tropomyosin 2 and ten vimentin isoforms).

The function of these proteins is reported in **table 7**.



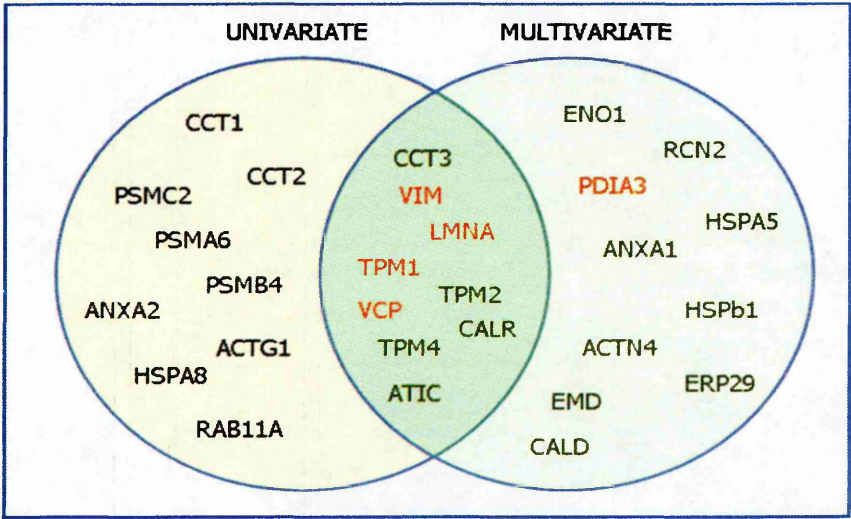
**Figure 40.** Expression profile cluster of the 42 discriminating spots for the PCA analysis of 10-days exposure untreated vs. TCDD 100nM (TCDD2) treated samples. There were 8 down-regulated protein species (blue) and 34 up-regulated proteins (red).

**Table 7.** Protein function

Symbol	Uniprot Swiss- Prot	Protein name	Protein Function
ACTN4	Q9QXQ0	Alpha-actinin-4	F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures.
ANXA1	P07150	Annexin A1	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.
ATIC	O35567	Phosphoribosylaminoimidaz olecarboxamide formyltransferase	Metabolic enzyme involved in the purine biosynthesis.
CALD	Q62736	L-caldesmon	Actin- and myosin-binding protein implicated in the regulation of actomyosin interactions in smooth muscle and nonmuscle cells. Stimulates actin binding of tropomyosin which increases the stabilization of actin filament structure. Interacts with actin, myosin, two molecules of tropomyosin and with calmodulin.
CCT3	Q6P502	T-complex protein 1 subunit gamma	Molecular chaperone; assist the folding of proteins upon ATP hydrolysis.
EMD	Q63190	Emerin.	Involved in stress resistance and actin organization.
ENO1	P04764	Alpha-enolase	Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses.
ERP29	P52555	Endoplasmatic reticulum protein 29	Plays an important role in the processing of secretory proteins within the endoplasmic reticulum (ER), possibly by participating in the folding of proteins in the ER.
HSPA5	P11021	Heat shock 70 kDa protein 5	Plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
HSPB1	P42930	Heat shock protein beta-1	Involved in stress resistance and actin organization.
LMNA	P48679	Lamin-A.	Component of the nuclear lamina, which is thought to provide a framework for the nuclear envelope and may also interact with chromatin.
PDIA3	P11598	Protein disulfide-isomerase A3	Endoplasmic reticulum lumen protein involved in protein importing into nucleus and protein retention in ER.
RCN2	Q62703	Reticulocalbin-2	Calcium-binding protein.
TPM1	P04692	Tropomyosin alpha-1 chain	Cytoskeleton element binding to actin filaments in muscle and non-muscle cells.
TPM2	P58775	Tropomyosin beta	Cytoskeleton element binding to actin filaments in muscle and non-muscle cells.
TPM4	P09495	Tropomyosin alpha-4	Cytoskeleton element binding to actin filaments in muscle and non-muscle cells.
VCP	P46462	Valosin-containing protein	Hexameric type II ATPase with multiple cellular activities.

This trend in the effect of TCDD exposure duration on the osteoblast proteome profile is in accordance with the conclusions from the univariate analysis (*see section 4.3*).

Cross-comparison of the protein spots highlighted by univariate and multivariate analysis found 47% proteins in common contributing to the discrimination of untreated and TCDD treated 10-day groups as shown in **figure 41**.



**Figure 41.** Venn diagram of osteoblast proteins expressed differentially after 10 days of TCDD exposure using two statistical approaches. In red are proteins with FDR<5% derived from multivariate analysis.

**4.4.1. Differential proteomic analysis using a multivariate approach for data assessment: remarks**

The PCA, which considers data globally, has been applied to dataset obtained from multi-gel experiments to assess whether TCDD treatment (doses and length of exposure) had any significant effect on the proteome profile of differentiating osteoblasts.

Four main conclusions can be drawn from the results obtained using this alternative statistical approach:

1. The PCA identified particularly robust changes in the proteome profile correlated to a duration based effect (culture days) rather than an exposure effect (TCDD doses). This conclusion was unique to the PCA.
2. Despite the relevant time-effect, PCA showed that the overall proteome profile was statistically distinguishable between untreated and treated cells only when cells were exposed to the highest dose of TCDD for 10 days. This finding was in accordance with the univariate analysis of the same dataset.
3. A number of protein spots (47%), contributing to the separation between 10-days untreated and TCDD treated group, were common to the two statistical approaches. These proteins highlighted by both univariate and multivariate statistical analyses are highly likely to be significant, despite the possibility of false-positives. The presence of proteins not shared by both analyses underline the different conceptual basis of the two methods.
4. The application of these two approaches to the same dataset demonstrated their complementary roles for identifying spots with expression changes. As PCA identifies correlated changes, it is particularly useful for recognizing pathway changes and events correlated to each other, whereas the univariate method is more appropriate for identifying protein expression changes that are significant, but not directly related to changes in other proteins detected in the sample.

#### 4.5. Confocal microscopy

In this project, I applied confocal microscopy to explore the role of TCDD on the adhesion properties of osteoblasts. Thus, it constituted a validation method for the proteomic findings, showing that cytoskeletal components were very susceptible to TCDD exposure.

*Confocal microscopy* offers several advantages over conventional wide field optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the ability to collect serial optical sections from thick specimens. The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare when specimen thickness exceeds the immediate plane of focus. Laser scanning confocal microscopy of biological materials relies heavily on fluorescence as an imaging mode, primarily because of its high sensitivity and ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues.

*Cell adhesion* to extracellular matrices provides the environmental cues necessary to control a cell's decision to survive, proliferate, differentiate or migrate. Cell-matrix interactions generate anchorage forces that mediate cell spreading and migration. Adhesion to extracellular matrix components, such as fibronectin and laminin, is primarily mediated by integrin receptors, as shown in **scheme 1**. Integrin-mediated adhesion is a highly regulated process involving receptor activation and mechanical coupling to extracellular ligands. Bound receptors rapidly associate with the actin cytoskeleton and cluster together to form *focal adhesions*, discrete supramolecular

complexes that contain structural proteins, such as vinculin and talin, and signalling molecules, including Focal Adhesion Kinases (FAC) and Src.

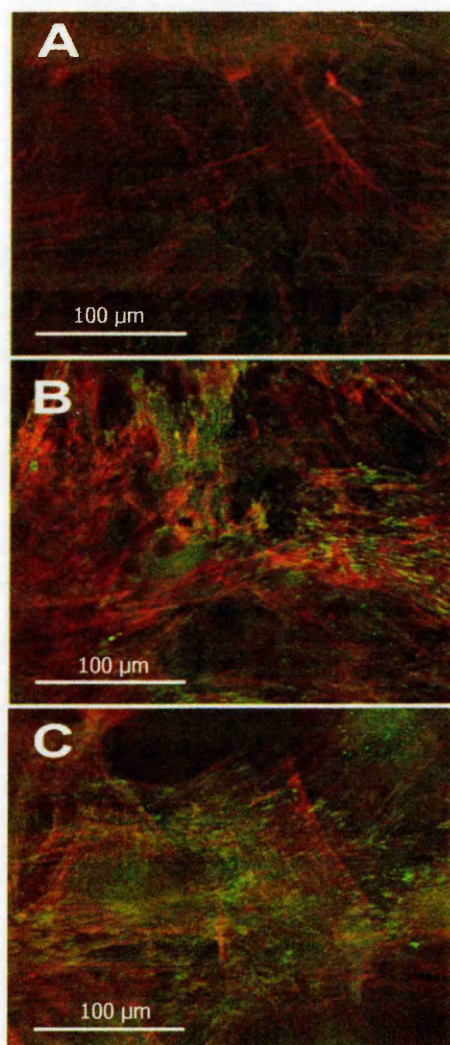
Confocal microscopy analysis was carried out in collaboration with Prof. Juha Tuukkanen at the Department of Anatomy and Cell Biology, Institute of Biomedicine, University of Oulu, Finland.

Cell adhesion was qualitatively analysed by immunofluorescence of proteins (actin and paxillin) participating in the attachment of cells to the substrate. Paxillin anchors the actin cytoskeleton and forms focal adhesions by linking actins to transmembrane integrins.

We examined osteoblasts double-labelled with fluorescently tagged actin to reveal the F-actin cytoskeleton and an anti-paxillin antibody to show focal contacts. The amount of paxillin (green spots) and its organization in focal adhesions were visually evaluated from the images.

As shown in **figure 42**, increased focal contact formation and a more robust stress fiber-containing actin cytoskeleton were evident in osteoblasts exposed to TCDD for 10 days. The intensity of the fluorescence and the fibrillar organization were much lower in controls (A) than exposed cells (B and C). No apparent difference was found between the two TCDD doses. **Figure 42** indicates typical microscope fields of each sample type.





**Figure 42.** Effects of TCDD on focal adhesion complexes (paxillin stained with ALEXA Fluor 488, green; and actin stress fibers with TRITC-conjugated phalloidin, red). Differentiating osteoblasts from rat bone marrow were either untreated (A), or treated with 1 nM TCDD (B) or 100 nM TCDD (C) for 10 days, then immunostained as described under Material and Methods. Pictures for each treatment reflect the typical appearance of the staining intensity and organization of the cytoskeleton.  
41x122mm (300 x 300 DPI)

#### ***4.5.1. Confocal microscopy: remarks***

Staining for the focal adhesion marker paxillin showed an increase in focal adhesion contacts in osteoblasts after 10 days of exposure to 100nM TCDD. This might imply enhanced adhesion of exposed osteoblasts, which could interfere with/or reduce the differentiation processes [193]. These effects on adhesion-mediated events agree with previous reports of alterations of cell plasticity and cell mobility after AHR activation [46, 193-195].

## 4.6. Pathway analysis

The functional classification and significance of differently expressed proteins was evaluated by adopting a bioinformatic approach of linking known proteins to their putative biological functions using the MetaCore mapping tool as previously described (section 3.15).

Attention has been mainly focused on the differentially expressed proteins highlighted by univariate and multivariate analysis in differentiating osteoblasts exposed for 10 days to TCDD.

Further interpretation of the proteomic data was carried out on the proteins whose abundance was significantly modulated by time differences (untreated osteoblasts culture days) and not by TCDD exposure (multivariate analysis).

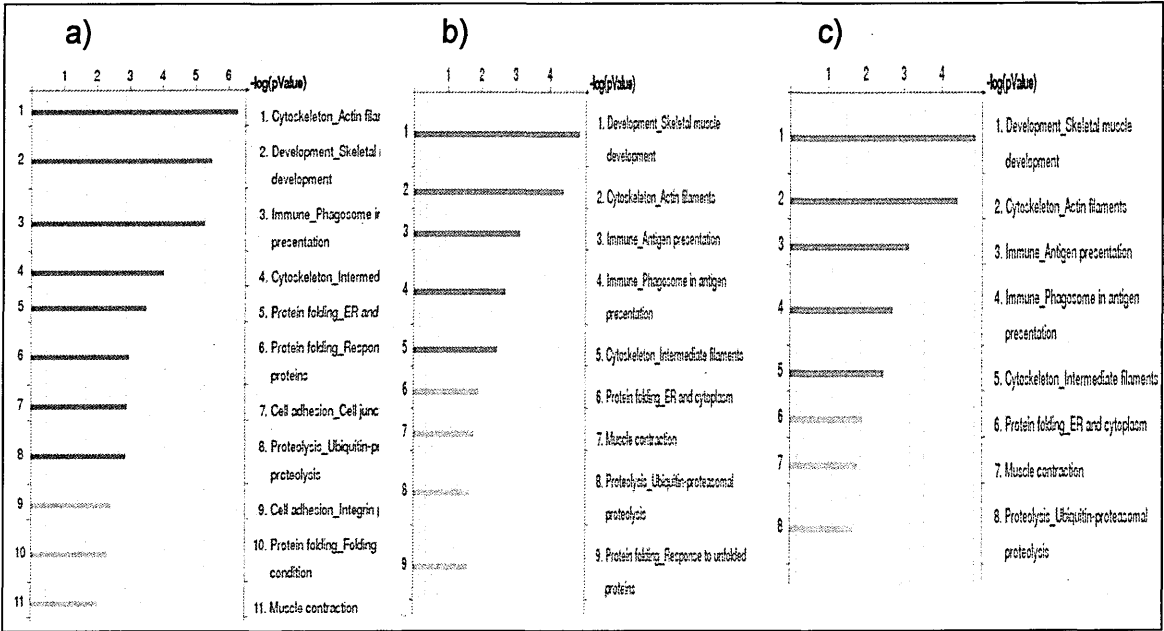
The MetaCore software became available in the laboratory in 2008, thus my proteomic data were the first training set used to generate networks and to interpret experimental data.

### 4.6.1. Functional ontology enrichment

The biological process enrichment was analysed based on GO Ontology processes. Both *univariate* and *multivariate analysis* showed that the differentially expressed proteins modulated by 10 days TCDD exposure were mostly involved in cytoskeleton organization, actin filament-based process, protein transport and folding, as shown in **figure 43 (panel A and B respectively)**.

The classification of biological processes associated with proteins whose expression was modulated only by the progress of osteoblastic differentiation (*multivariate*

analysis) confirms that cytoskeletal components and protein folding processes were mostly involved (figure 43, panel C).



**Figure 43.** Biological processes associated with differently expressed proteins in unexposed and 10 days TCDD-exposed differentiating osteoblasts from **a)** univariate analysis; **b)** multivariate analysis. Panel **c)** refers to biological processes associated with differentially expressed proteins related to the progress of unexposed osteoblast differentiation. Histograms of the negative log of each p-value for the experimental groups ( $p < 0.05$ ) are shown..

4.6.2. Biological Network analysis

Further functional interpretation of the differentially expressed proteins was carried out by building and analyzing biological networks.

Using the shortest path algorithm to map the shortest paths of interaction among the differentially expressed proteins, 16 out of the 18 submitted proteins highlighted by 10 days of TCDD exposure (*univariate analysis*), were brought together in the network of interactions (figure 44).

Two prominent regulatory proteins in the network were *c-Fos* and *c-Myc*. These interact directly with respectively three (RAB11A, lamin A, and vimentin) and four (lamin A, HSPA8, tropomyosin 2, and gamma actin) of the differently expressed proteins. Other transcription factors such as p53 and c-Jun were also identified in the networks, suggesting a common role in driving TCDD's effects in osteogenesis.

The relevance of these transcription factors was further highlighted when proteomic data derived from the *multivariate analysis* were analyzed. In this case 12 out of the 18 identified proteins were clustered together in the interaction network (**figure 45**). Generation of sub-networks centered on transcription factors revealed that *c-Myc* and *c-Fos* were directly associated with the transcription regulation of 6 proteins (VCP, calreticulin, tropomyosin 2,  $\alpha$ -enolase, lamin A, and vimentin) (**figure 46**, upper network).

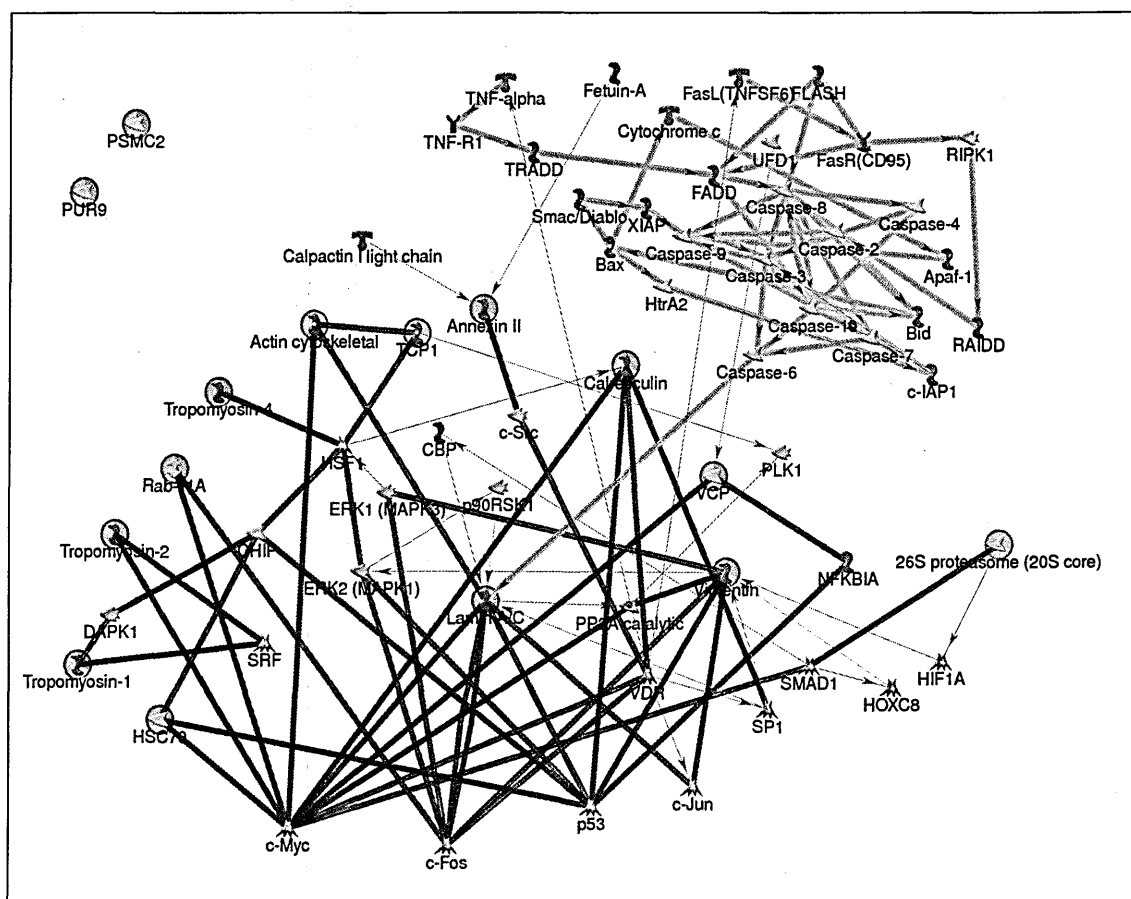
Interestingly, *c-Myc* and p53 had a prominent role in the regulation of 11 proteins (tropomyosin 2, ACON=ACO2, EEF1G,  $\alpha$ -enolase, lamin A calreticulin, ARF1, VIM, HSP27=HSPB1, ERP29) whose expression gradually changed as osteoblasts differentiated (**figure 46**, lower network).

#### **4.6.3. Pathway analysis: remarks**

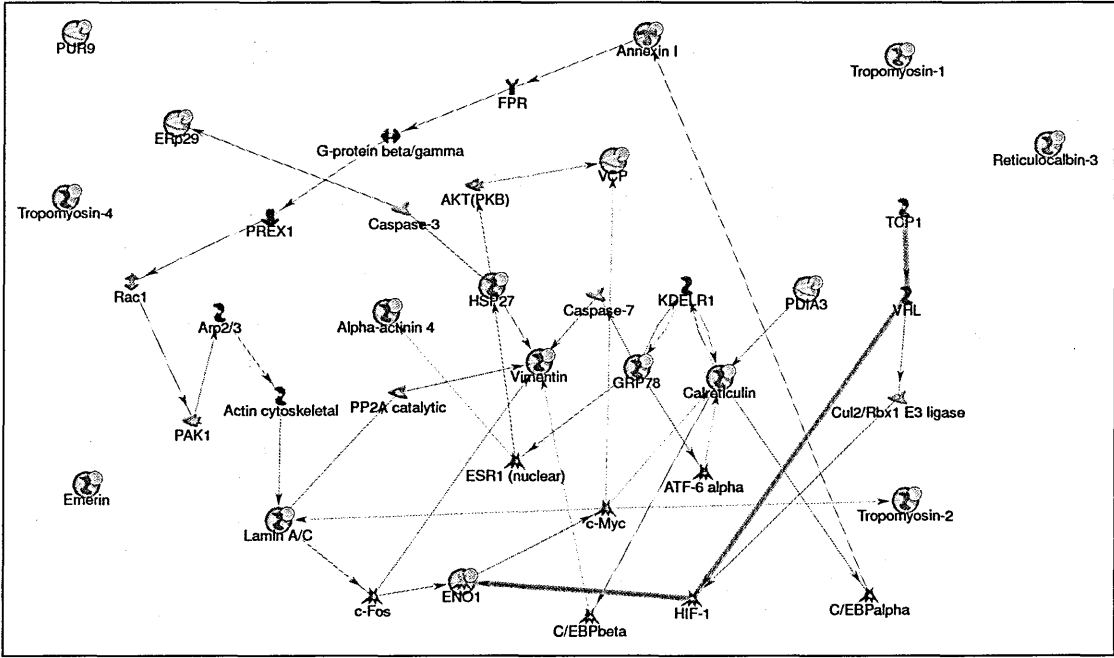
The biological processes enrichment analysis indicate that TCDD affects mainly osteoblasts cell architecture and trafficking proteins, essentially related to cell motility, organization and survival.

The integration of the experimental results with pathway analysis identified transcriptional factors such as *c-Fos* and *c-Myc* as prominent factors that might participate in the transcriptional regulation of some of the proteins whose abundances were changed by TCDD.

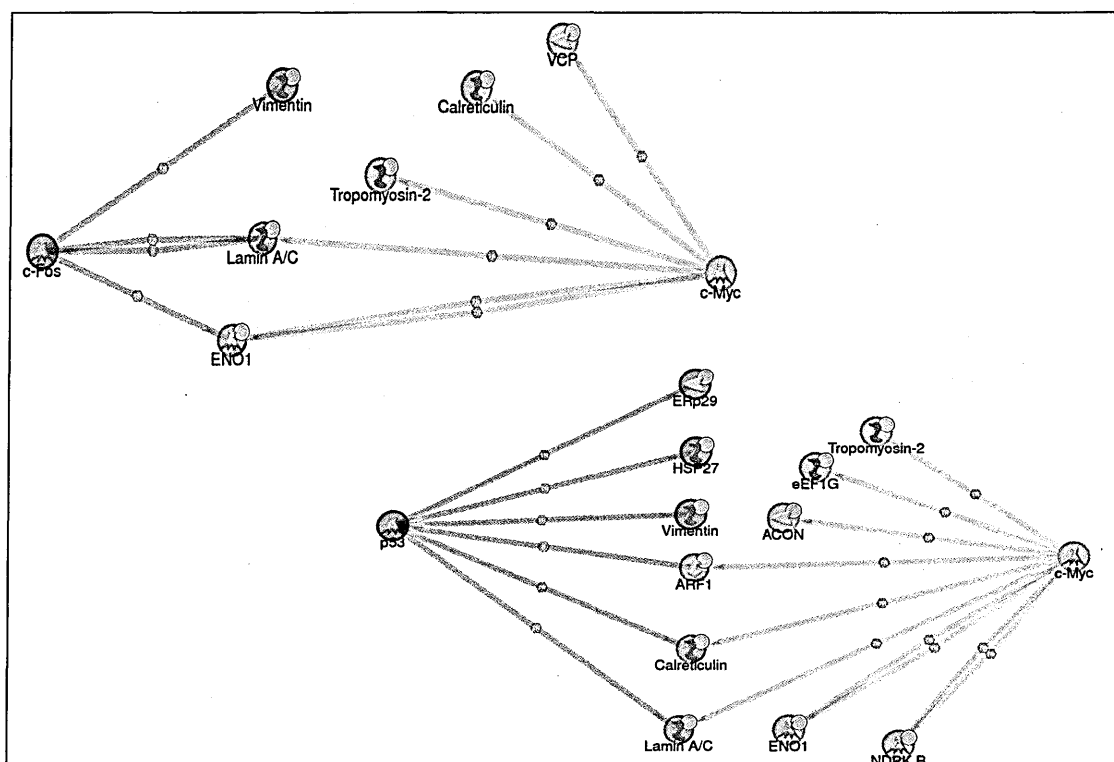
Additionally, the role of *c-Myc* and p53 as regulating factors in the proteome profile changes during osteoblasts differentiation provides clues to the complex interplay between TCDD's action and the molecules driving osteoblasts differentiation.



**Figure 44.** Biological network analysis of differently expressed proteins in differentiating osteoblasts in response to 10 days of TCDD exposure (univariate analysis). *Nodes* represent proteins. *Coloured circles* represent a single protein or a group of proteins identified in our analysis. The 26S proteasome (20S core) node includes PSMA6 and PSMB4; the TCP1 node represents CCT1, CCT2, and CCT3. PUR9 refers to ATIC, HSC70 refers to HSPA8. See Appendix 7.2 for full protein names. *Lines connecting the nodes* indicate interactions of activation or induction or modification or direct binding. The direct connections relevant for the identified proteins are traced in blue. The shortest path network was edited by hiding some nodes and interactions for the sake of clarity.



**Figure 45.** Biological network analysis of differently expressed proteins in differentiating osteoblasts in response to 10 days of TCDD exposure (multivariate analysis). *Nodes* represent proteins. *Coloured circles* represent a single protein or a group of proteins identified in our analysis. PUR9 refers to ATIC, the TCP1 node represents CCT3, GRP78 refers to HSPA, and HSP27 refers to HSPB1. See Appendix 7.2 for full protein names. *Lines connecting the nodes* indicate interactions of activation or induction or modification or direct binding. The shortest path network was edited by hiding some nodes and interactions for the sake of clarity.



**Figure 46.** Transcription regulation networks. Nodes represent proteins. Coloured circles represent a single protein or a group of proteins identified in our analysis. Lines connecting the nodes indicate interactions of activation or induction or direct binding. *Upper network:* interactions between the transcriptional factors *c-Fos* and *c-Myc* and the subset of proteins whose abundance changed in differentiating osteoblasts after 10 days of TCDD exposure. *Lower network:* interactions between the transcriptional factors *c-Myc* and *p53* and the subset of proteins whose abundance changed during the progress of osteoblast differentiation. ACON refers to ACO2, NDPK B refers to NME2, and HSP27 refers to HSPB1. See **Appendix 7.2** for full protein names.

**CHAPTER 5**  
**CONCLUDING DISCUSSION**



Dioxins are ubiquitous environmental contaminants and immunotoxicants that are persistent, lipophilic, and accumulate in the food chain. While a broad range of dioxin toxicities have been documented, their effects on bone are still poorly understood.

Only a few experimental studies have demonstrated that bone development and homeostasis are targets for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is the most potent model compound for dioxins. TCDD was reported to interfere with bone growth modelling and mechanical strength *in vivo*, at doses relevant for environmental health risk assessment. Data from *in vitro* studies showed that TCDD suppresses differentiation of normal diploid rat osteoblasts.

Despite these earlier studies, the detailed mechanisms of TCDD-induced effects on bone are still unknown.

Using a proteomic approach, this study investigated the disturbances of bone development caused by TCDD, focusing on osteoblastogenesis. I chose the differentiation of rat mesenchymal stem cells to osteoblasts as a model system to compare protein expression in untreated and TCDD-exposed cells over three time points (3, 7, and 10 days of culture, covering the main phases of osteogenesis) at two dioxin dose levels. The two doses (1nM and 100nM) were in the range of those used in the literature for *in vitro* cell systems, sufficiently low to avoid any block in cell proliferation, but high enough to induce the AHR-dependent mechanism [123].

I set out to determine whether TCDD affected protein abundance and how these effects correlated with alterations in biochemical indices of osteoblast activity and differentiation, to gain further insight into the molecular mechanisms involved in TCDD's effect on osteoblast differentiation.

My results indicate that differentiating osteoblasts are sensitive to TCDD as measured by mRNA expression of *differentiation markers* such as Runx2 (a transcription factor

essential for osteoblast differentiation), alkaline phosphatase (Alp, a cell-surface glycoprotein whose expression is acquired during matrix maturation) and osteocalcin (Ocn, a matrix protein synthesized by osteoblasts and used as a biomarker for bone formation).

The gene expression analysis indicated no change in markers expression at earlier time points of TCDD exposure, when cells are still proliferating (3 days) or when they enter the differentiation stage (7 days). After 10 days, at which point the cells should be fully differentiated osteoblasts, expression of all measured markers was significantly decreased, with the strongest inhibitory effect on the expression of osteocalcin.

These results clearly indicate time-dependent TCDD-induced changes in osteoblast marker expression, suggesting that TCDD inhibits osteoblast differentiation *in vitro* and supporting the anti-osteogenic potential of dioxin.

TCDD is known to mediate its effects mainly via AHR, by binding to the dioxin response element (DRE) located upstream of the target genes. Immunofluorescent staining indicated that the cells used in this study actually expressed AHR from the beginning of the exposure period, and that upon TCDD exposure AHR was translocated to the nucleus (experiments performed at the University of Oulu, Prof. Juha Tukkanen; data not shown). However, Alp and Ocn genes both lack DREs in their regulatory sequences. Therefore, the effect of TCDD on the expression of these genes is probably not a direct effect, but rather a secondary response mediated by AHR.

This prompted me to apply a *proteome approach* to my model system, in order to explore changes in the cellular expression of proteins that may be important signatures of TCDD-associated signalling events influencing osteoblast differentiation.

I have shown that at early time points of TCDD exposure (3 and 7 days) TCDD had no relevant effect on the >700 polypeptides that could be routinely analysed. The only exception was the significant decreased expression of calregulin occurring after 7 days

of TCDD treatment. Calregulin is a chaperonin protein that regulates  $\text{Ca}^{2+}$  levels by modulation of the endoplasmatic reticulum (ER)  $\text{Ca}^{2+}$  storage and transport [178]. Thus, the reduction of calregulin might represent an early marker of the TCDD-induced calcium homeostasis disruption, since calregulin is also down regulated following 10 days of exposure, at which time I showed calcium deposition was decreased in TCDD-exposed osteoblasts.

The most significant rearrangement of the proteome during osteoblast differentiation was observed following the longer time of TCDD exposure (10 days), at which time mRNA changes in differentiation markers were also observed. Eighteen individual proteins were changed in abundance mainly at the highest dose of TCDD, indicating dose- and time-dependent changes in protein expression.

This differential proteomic analysis was performed using a *univariate statistical approach*, which is currently the conceptually simplest and the most commonly used in gel differential expression analysis.

A distinctive aspect of my work was the additional use of an alternative statistical approach to the same dataset. In this case I applied *principal component analysis (PCA)* as an exploratory data analysis method able to visualize differences between complex proteome profiles.

The PCA identified particularly robust changes in the proteome profile correlated to a time based effect (culture days) rather than an exposure effect (TCDD doses). Despite this relevant time-effect, the PCA showed that the overall proteome profile was statistically distinguishable between untreated and treated cells only when cells were exposed to the highest dose of TCDD for 10 days. This finding was in accordance with the conclusion from the univariate approach applied to the same dataset. Furthermore both statistical approaches shared a fair number of proteins that are highly likely to be significant in the biological process investigated.

At 10 days of exposure, TCDD strongly induced the expression of cytoskeletal components such as tropomyosin, vimentin and gamma actin. All these proteins are implicated in one or more steps of cytoskeleton protection, stress fiber assembly and cell motility [163, 179].

This evidence prompted me to further explore the effects of TCDD on cell architecture by confocal microscopy. Staining for the focal adhesion marker paxillin showed an increase in focal adhesion contacts in osteoblasts after TCDD treatment. This implies enhanced adhesion of exposed osteoblasts, which might interfere with/or reduce the differentiation processes [193]. Such effects on adhesion-mediated events agree with previous reports of alterations of cell plasticity and cell mobility after AHR activation [46, 193, 195].

Since cell differentiation depends on appropriate attachment to adjacent cells and/or extracellular matrix [196], it appears plausible that TCDD's negative effect on osteoblast differentiation might be related to dioxin interfering with signalling pathways regulating cell-cell and cell-substrate adhesion and, by extension, with the transmembrane signals important for cell organization and motility.

Cell adhesion and spreading are calcium-regulated, involving a plethora of calcium-binding proteins. One such protein is calregulin. As already mentioned, this multifunctional protein participates in calcium homeostasis through its calcium storage capacity, in protein "quality control" through its chaperonin activity and in cell adhesion through pathways that are still not clear [182]. It has been reported that fibroblasts under-expressing calregulin have a poorly adhesive phenotype [197]. However, in the present study TCDD-exposed osteoblasts showed a decrease in the abundance of calregulin, with concomitant enhanced adhesiveness. This suggests that TCDD-induced changes in osteoblast adhesion properties cannot be simply correlated with the differential expression of calregulin, but are probably integrated in additional signalling pathways that control cell adhesion.

Interestingly, after 10 days of exposure, TCDD down-regulated another calcium-binding protein, annexin A2, which functions as a  $\text{Ca}^{2+}$  channel in the plasma membrane and in the matrix vesicles, which initiate mineralization in bone [183]. Under-expression of annexin A2 might therefore interfere with calcium deposition, and indeed it was significantly decreased after 10 days of exposure to TCDD in my cell system.

Further important pathways influenced by 10 days TCDD treatment are related to protein quality control and trafficking. These are both vital for osteoblasts and polarized cells, where high levels of synthesis of secreted proteins require a highly evolved mechanism to fold, process and secrete proteins properly. Evidence is emerging that tight control and coordination of the endoplasmic reticulum stress response is indispensable to ensure proper function and survival of professional secretory cells like osteoblasts [198].

In osteoblasts exposed to TCDD for 10 days there was a significant decrease in the expression of chaperonins containing T-complex polypeptides 1, 2 and 3 which assist in the folding of actin and tubulin, suggesting deregulation of the protein-folding regimen with consequences in cytoskeleton organization.

The intracellular trafficking machinery of osteoblasts is also likely to be impaired by the down-regulation of RAB11A, a member of the large superfamily of small Ras-related GTP-binding Rab proteins, acting as master signal components that regulate many aspects of vesicular transport along recycling, endocytic and exocytic pathways. Since the maintenance of polarity is largely dependent on endocytic sorting [199, 200], impaired protein trafficking control is likely to have dramatic effects on osteoblast secretory activity.

In this scenario, the TCDD-strengthened expression of PSMC2, a 26S subunit regulatory unit, and of PSMB4, the 20S catalytic/enzymatic core, together with the down-regulation of PSMA6, the alpha-type subunit of the 20S proteasome, although

apparently contradictory, might be simply a sign of changes in the proteasome subunit composition and activity after exposure to TCDD.

It might be therefore speculated that TCDD disrupts protein folding efficiency and trafficking control, impairing the functionality of osteoblasts.

The TCDD-induced up-regulation of the chaperonin HSPA8 can be viewed as an adaptive response of the cell to reinforce the protein-folding control process and to boost tolerance to stressful conditions.

I found that 10-days of TCDD treatment enhanced the abundance of valosin-containing protein, a general chaperone that targets many substrates to the proteasome for degradation [201]. Valosin-containing protein is implicated in a large number of biological functions and it may exert an anti-apoptotic effect [201, 202]. This is consistent with the lower Bax/Bcl-2 expression ratio I found in TCDD-exposed osteoblasts by Western blot analysis. This suggests that TCDD might reduce the apoptotic ability of differentiating osteoblasts. Apoptosis increases with osteoblast maturation *in vitro* and is a prominent feature during the development of the mature osteoblast phenotype, removing populations of cells that would otherwise inhibit differentiation [203]. Therefore apoptotic inactivation by TCDD may have detrimental effects on the efficiency of osteoblast differentiation.

The attenuation of the osteoblast differentiation process might also be associated with the strong down-regulation of lamin-A and all its isoforms I observed in the proteome profile of TCDD-exposed osteoblasts. Lamin-A, a major structural component of the nuclear envelope, is considered critical for cell differentiation probably by "locking" cells in a specific phenotypic stability [187]. Its decreased expression might therefore interfere with the maintenance of the osteoblasts differentiated state. It has been shown that inhibition of lamin-A in osteoblastic lineage cells impairs osteoblastogenesis [204].

Interestingly, long-term (10 days) TCDD exposure induced changes in the expression of these proteins that were opposite to the expression trend observed when cells were grown in osteogenic conditions without dioxin.

The up-regulation of calregulin observed during the process of osteoblast differentiation is consistent with the reported increase in calregulin expression accompanying the differentiation of mouse bone marrow osteoprogenitor cells into preosteoblast cells upon parathyroid hormone treatment [185]. It may thus participate in an intracellular  $\text{Ca}^{2+}$  transport pathway and could act as a  $\text{Ca}^{2+}$  carrier to the mineralization front. Such role would be jeopardized by TCDD, which might impair the osteoblast mineralization process reducing the level of calregulin.

I found that TCDD also abolished the expression trends of annexin A2, lamin-A, RAB11A and chaperonins CCT1, CCT2, CCT3. These proteins showed a gradual up-regulation during differentiation, in accordance with their functional role in growth and differentiation.

The drop of their expression levels caused by TCDD is likely to impair the osteogenic progression and osteoblast function by lowering their secretory capacity, ability to maintain the differentiated state, protein folding efficiency and trafficking control.

During osteogenic differentiation, MSCs change from their characteristic fibroblast-like phenotype to a near spherical shape that characterizes the differentiated state, with a consequent change in assembly and disassembly kinetics of microfilaments dynamics [188].

In my model system, osteoblast differentiation was accompanied by a gradual decrease in the expression of tropomyosin, a major microfilament stabilizing component, consistent with the tropomyosin reduction reported in differentiating systems of non-muscle cells [189, 190]. In parallel I observed a differential expression of type III intermediate filament vimentin, which was reduced after 10 days of culture,

when the majority of cells have changed their shape to a more rounded differentiated stage [185, 190].

Such expected changes in cytoskeletal organization during the osteogenic differentiation were again abolished by 10 days of TCDD exposure, which caused enhanced expression of all tropomyosin isoforms and vimentin.

Taken together these results indicate that, within 10 days of exposure, TCDD induces concerted changes in expression in the differentiating osteoblasts proteome that are in the opposite direction of the trend characterizing osteoblast differentiation. The observation that this effect occurs following 10 days of culture might indicate that later stages of differentiation, essential for both the development and maintenance of bone, might be particularly susceptible to TCDD.

Some of the proteome alterations observed after TCDD exposure in differentiating osteoblasts may represent specific novel targets of TCDD on cells, since it has been recently reported that, in rat hepatoma cells, TCDD affects the expression of cytoskeletal-associated proteins such as vimentin, gamma actin, tropomyosins, ANXA2, lamin-A, molecular chaperonin HSPA8, T-complex protein subunits CCT1-3 and protein degradation proteins [205]. They may therefore provide novel starting-points for exploring specific aspects of dioxin toxicity in mammalian cells.

The integration of my proteomics results with *pathway analysis* identified transcriptional factors such as *c-fos* and *c-myc* as prominent nodes. These factors might participate in the transcriptional regulation of some of the osteoblast proteins whose abundances were changed by TCDD. HSPA8, tropomyosin 2 and gamma actin can be genomic targets of the *c-myc* protein [206]. In addition, the expression of RAB11A and vimentin depend on *c-fos* [207, 208], while lamin-A might interact with *c-fos* and disturb bone development and homeostasis through as yet unknown mechanisms [209]. Previous studies have demonstrated a critical role for *c-fos* in



osteoblast responses to hormones and in bone matrix formation, suggesting that down-regulation of *c-fos* expression might be translated into a decrease in osteoblast differentiation capability [210]. Furthermore *c-fos* is important in gene transcription of osteogenic markers, such as Ocn and Alp [211], that were down-regulated by TCDD in this study. Finally, the reported ability of TCDD to interfere with both *c-myc* and *c-fos* DNA-binding activity and expression is of relevance [212, 213].

Further investigations are warranted to assess the involvement of these early-response genes. Their modulation is likely to play a major role in early molecular events surrounding TCDD-induced inhibition of osteoblast differentiation. This suggests that TCDD might produce effects on the osteoblast proteome through alteration of signal transduction mechanisms rather than by changing the abundance of key structural proteins.

The dioxin-responsive protein targets found in this study might be the downstream effect of the interactions between stress-activated pathways involved in dioxin's action and bone cell signalling pathways.

In conclusion, the results of this work indicate that TCDD effects the differentiation of osteoblasts by altering their cell architecture, adhesive properties and calcium homeostasis, causing an abnormal trafficking and targeting of proteins and thereby interfering with signals important for cell motility, organization and survival.

Additionally, integrating my experimental results with pathway analysis, I provide clues to the complex interplay between TCDD's action and the regulatory molecules that alter signal-transduction pathways regulating osteoblast differentiation.

From these observations, it is clear that the proteomics approach I used to explore the effect of TCDD on osteoblast differentiation is a '*hypothesis-generating engine*' rather than an hypothesis-testing platform. It delivers compelling hypotheses that help in

explaining the molecular mechanisms by which endocrine-disrupting chemicals, such as dioxins, interfere with bone development, quality and diseases.

I am aware that focused follow-up studies that test my emerging hypotheses directly are needed to provide a framework for translating my data into an understanding of complex biological processes.

**CHAPTER 6**  
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**CHAPTER 7**  
**APPENDIX**

### 7.1. List of abbreviations

*AR* activity rate

*EF* emission factor (average emission rate of a given pollutant for a given source, relative to the intensity of a specific activity)

*I-TEQ* international toxic equivalent

*MSW* municipal solid waste

### ***Protein abbreviations***

ACTG1: gamma-actin;

AHR: aryl hydrocarbon receptor;

Alp: alkaline phosphatase;

ANXA2: annexin II;

ATIC: phosphoribosylaminoimidazolecarboxamide formyltransferase;

BAX: apoptosis regulator BAX;

BCL2: apoptosis regulator Bcl-2;

CALR: calregulin;

CCT1: T-complex protein subunit alpha;

CCT2: T-complex protein 1 subunit beta;

CCT3: T-complex protein 1 subunit gamma;

GAPDH: glyceraldehyde-3-phosphate dehydrogenase;

HSPA8: heat shock cognate protein 71 kD;

LMNA: lamin-A;

Ocn: osteocalcin;

PSMA6: 20S proteasome subunit alpha type 6;

PSMB4: 20S proteasome subunit beta type 4;

PSMC2: 26S protease regulatory subunit 7;

RAB11A:	ras related protein Rab-11A;
RAN:	GTP-binding nuclear protein Ran;
Runx2:	runt-related transcription factor 2;
TCDD:	2,3,7,8-Tetrachlorodibenzo-p-dioxin;
TPM1:	tropomyosin-1 alpha chain;
TPM2:	tropomyosin beta chain;
TPM4:	tropomyosin alpha-4 chain;
VCP:	valosin-containing protein;
VIM:	vimentin.

## 7.2. List of publications

Pastorelli R, Saletta F, Carpi D, Campagna R, dell'Osta C, Schiarea S, Vineis P, Airoldi L, Matullo G. *Proteome characterization of a human urothelial cell line resistant to the bladder carcinogen 4-aminobiphenyl*. Proteome Sci. 2007 May 3;5:6.

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Pastorelli R, Carpi D, Airoldi L, Chiabrando C, Bagnati R, Fanelli R, Moverare S, Ohlsson C. *Proteome analysis for the identification of in vivo estrogen-regulated proteins in bone*. Proteomics. 2005 Dec;5(18):4936-45.

7.3. Protein Identification

SUPPLEMENTAL TABLE 1 on peptide identification

spot #	Identified protein	symbol	Uniprot-Swiss-Prot #	Score	# valid ident	# unique ident	CyG %	sequence	charge	m/z	d m/z	z-score	P-value	Pos	#MC	Modif
8	Lamin-A	LMNA	P48679	258.6	38	25	42	R/SVGGSGGSGGNLVTR/S	2	783.96	-0.081	14.1	5.17E-42	629-645	0	.....
								R/TALINATGEVAMR/K	2	737.97	0.41	13.7	5.27E-40	528-541	0	.....
								R/TALINATGEVAMR/K	2	738.45	-0.07	13.7	4.85E-40	528-541	0	.....
								R/TALINATGEVAMR/K	2	738.47	-0.09	13.4	8.58E-38	528-541	0	.....
								R/SGAASSTPLSPTR/I	2	680.55	-0.203	12.3	9.71E-32	Dec-25	0	.....
								R/TALINATGEVAMR/K	2	746.42	-0.043	12.2	2.81E-31	528-541	0	.....Oxidation M::
								R/NSLVGAHHELOOSR/I	2	877.01	-0.075	12.2	3.54E-31	281-296	0	.....
								R/TVLGTCGQPADK/A	2	703.92	-0.086	12	2.74E-30	587-599	0	.....Cys CAM::Cys CAM::.....
								R/TITSEEVSR/E	2	574.9	-0.106	12	4.22E-30	63-72	0	.....
								R/TDSLISAOISLOK/O	2	716	-0.104	11.7	8.21E-29	299-311	0	.....
								R/VAVEVDDEGK/F	2	602.43	-0.139	11.3	1.02E-26	440-450	0	.....
								R/GSHCSSGDPAEYLR/S	2	868.97	-0.101	11.2	2.64E-26	569-584	0	.....Cys CAM::.....
								R/EAALSTALSEK/R	2	560.44	-0.142	11.1	9.17E-26	145-155	0	.....
								R/SLFENAGLR/L	2	545.4	-0.119	10.9	1.73E-24	51-60	0	.....
								R/LOEKEDLOELNDR/L	2	815.48	-0.072	10.3	7.33E-22	29-41	1	.....
								R/TLEGELHDLR/G	2	592.03	-0.22	10.3	5.08E-22	157-166	0	.....
								R/TITSEEVSR/E	2	574.93	-0.136	10.1	5.96E-21	63-72	0	.....
								R/TLEGELHDLR/G	2	592.06	-0.25	10.1	3.89E-21	157-166	0	.....
								R/SLFENAGLR/L	2	545.39	-0.109	9.79	1.20E-19	51-60	0	.....
								R/LADALOELR/A	2	514.99	-0.199	9.67	4.19E-19	241-249	0	.....
								R/MOOLDEYOELLDIK/L	2	955.58	-0.116	9.52	1.14E-18	352-366	0	.....Oxidation M::.....
								R/KINYSEELR/E	2	512.39	-0.131	9.4	5.12E-18	209-216	0	.....
								R/LAVYDTRV	2	425.39	-0.145	8.81	1.26E-15	42-48	0	.....
								R/LEALGEAK/K	2	451.41	-0.157	8.56	1.45E-14	172-180	0	.....
								R/AAAYAEALGDAR/K	2	583.4	-0.122	8.53	1.31E-14	79-89	0	.....
								R/LVEDNGK/O	2	444.34	-0.094	8.5	2.30E-14	226-233	0	.....
								R/KLEALLNSK/E	2	501.91	-0.133	8.49	2.09E-14	136-144	0	.....
								R/KEDLLAAR/L	2	522.4	-0.122	7.94	2.15E-12	124-133	0	.....
								R/ENAEWRARMOOLDEYOELLDIK/L	3	956.27	0.524	7.93	1.54E-12	344-366	2	.....
								R/LADALOELR/A	2	514.87	-0.079	7.89	3.31E-12	241-249	0	.....
								R/LAVYDTRV	2	425.35	-0.105	7.87	3.71E-12	42-48	0	.....
								R/SGAASSTPLSPTR/I	2	680.94	-0.593	7.78	6.53E-12	Dec-25	0	.....
								R/LSPSTSOR/S	2	486.81	-0.051	7.63	2.39E-11	389-397	0	.....
								R/LOELSKV	2	415.85	-0.097	7.27	4.25E-10	102-108	0	.....
								R/LOELSKV	3	544.07	-0.129	6.65	3.55E-08	29-41	1	.....
								R/LOELSKV	2	415.91	-0.157	6.18	7.34E-07	102-108	0	.....
								R/TLEGELHDLR/G	3	394.92	-0.045	6.15	1.03E-06	157-166	0	.....
								R/AAAYAEALGDAR/K	1	1165.66	-0.112	6.13	3.78E-07	79-89	0	.....
10	Lamin-A	LMNA	P48679	139	19	15	25	R/TALINATGEVAMR/K	2	738.45	-0.07	12.9	6.69E-35	528-541	0	.....
								R/TALINATGEVAMR/K	2	746.43	-0.053	12.6	2.13E-33	528-541	0	.....Oxidation M::
								R/SGAASSTPLSPTR/I	2	680.4	-0.053	12	3.30E-30	Dec-25	0	.....
								R/SLTMVEDDDDEEDGELLH/H	2	1153.03	-0.061	12	2.29E-30	546-565	half	.....
								R/SVGGSGGSGGNLVTR/S	2	783.92	-0.041	11.4	3.71E-27	629-645	0	.....
								R/TITSEEVSR/E	2	574.88	-0.086	11.3	8.15E-27	63-72	0	.....
								R/SLFENAGLR/L	2	545.34	-0.059	9.81	1.06E-19	51-60	0	.....
								R/AAAYAEALGDAR/K	2	583.37	-0.092	9.09	8.91E-17	79-89	0	.....
								R/VAVEVDDEGKVR/L	2	803.48	-0.07	8.43	2.67E-14	440-453	1	.....
								R/LADALOELR/A	2	514.87	-0.079	8.11	5.29E-13	241-249	0	.....
								R/LSPSTSOR/S	2	486.8	-0.041	8.01	1.24E-12	389-397	0	.....
								R/SLTMVEDDDDEEDGELLH/H	3	814.76	-0.092	7.65	1.56E-11	546-566	half	.....
								R/MOOLDEYOELLDIK/L	2	955.58	-0.116	7.59	2.14E-11	352-366	0	.....Oxidation M::.....
								R/SLTMVEDDDDEEDGELLH/H	3	820.09	-0.091	7.4	9.86E-11	546-566	half	.....Oxidation M::.....
								R/SGAASSTPLSPTR/I	2	680.91	-0.563	7.28	2.80E-10	Dec-25	0	.....
								R/LOELSKV	2	415.83	-0.077	6.96	3.98E-09	102-108	0	.....
								R/KINYSEELR/E	2	512.34	-0.081	6.63	3.20E-08	209-216	0	.....
								R/KEDLLAAR/L	2	522.35	-0.072	6.21	5.58E-07	124-133	0	.....
								R/LOELSKV	1	830.56	-0.062	6.02	1.01E-06	102-108	0	.....



spot	Calregulin	CAUR	P18418	131.8	37	16	53	K/DNSQVSGSLEDWDFLPPK/K	2	1196.12	-0.067	12.9	1.62E-35	186-206	0	.....	.....
11								K/DNHGDSYNIIMFGPDICGPGTK/K	3	814.68	-0.335	12.7	3.33E-34	121-142	0	.....	.....Oys CAM:.....
								K/DNSOVSESSLEDWDFLPPK/K	2	1196.16	-0.107	11.9	6.96E-30	186-206	0	.....	.....Oys CAM:.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	814.35	-0.005	11.4	1.92E-27	121-142	0	.....	.....Oys CAM:.....
								K/DNSOVSESSLEDWDFLPPK/K	2	1196.1	-0.047	10.8	1.79E-24	186-206	0	.....	.....Oys CAM:.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	819.08	0.597	10.5	4.68E-23	121-142	0	.....	.....Oys CAM:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	987.81	-0.374	9.74	1.14E-19	249-272	0	.....	.....
								K/EOFLDGDWNR/W	2	726.39	-0.059	9.38	5.83E-18	25-36	0	.....	.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	992.52	0.248	9.23	1.45E-17	249-272	0	.....	.....Oxidation M:.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	820.09	-0.413	8.84	2.01E-15	121-142	0	.....	.....Oys CAM:.....
								K/FYGDQKXGLQTSODAR/F	2	1043.65	-0.154	8.8	2.32E-15	56-73	2	.....	.....
								K/SGTIFDNFLTNDAYAEFGNETWGT/K/A	3	1090.3	-0.13	8.45	1.57E-14	323-351	0	.....	.....
								K/HEONIDCGGYK/L	2	738.88	-0.049	8.44	2.71E-14	99-111	0	.....	.....Oys CAM:.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	825.04	-0.032	8.34	5.87E-14	121-142	0	.....	.....Oxidation M:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	987.83	-0.394	8.27	7.40E-14	249-272	0	.....	.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	819.66	0.017	7.96	1.42E-12	121-142	0	.....	.....Oys CAM:.....
								K/EOFLDGDWNR/W	2	726.32	0.011	7.95	1.48E-12	25-36	0	.....	.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	992.82	-0.052	7.82	2.80E-12	249-272	0	.....	.....Oxidation M:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	992.81	-0.042	7.62	1.38E-11	249-272	0	.....	.....Oxidation M:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	595.64	-0.02	7.26	4.02E-10	208-222	1	.....	.....
								K/NVINK/D	1	700.46	-0.025	6.93	2.84E-09	154-159	0	.....	.....
								K/SGTIFDNFLTNDAYAEFGNETWGT/K/A	2	1636.31	-1.558	6.87	2.19E-09	323-351	0	.....	.....
								A/DPAITK/E	1	852.97	0.475	6.72	9.17E-09	18-24	half	.....	.....
								A/DPAITK/E	2	427.24	-0.013	6.69	1.91E-08	18-24	half	.....	.....Oxidation M:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	992.17	0.598	6.67	1.38E-08	249-272	0	.....	.....
								A/DPAITK/E	1	853.42	0.025	6.63	6.52E-08	18-24	half	.....	.....
								K/FVLSGGK/F	1	737.44	-0.021	6.58	2.08E-08	49-55	0	.....	.....
								K/FYGDQKXGLQTSODAR/F	3	696	-0.001	6.52	6.32E-08	56-73	2	.....	.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	988.14	-0.704	6.52	1.30E-07	249-272	0	.....	.....
								R/FYALSAR/F	2	414.18	0.044	6.41	1.27E-07	74-80	0	.....	.....
								K/DNHGDSYNIIMFGPDICGPGTK/K	3	820.05	-0.373	6.38	1.37E-07	121-142	0	.....	.....Oxidation M:.....
								K/KPDDWDEMDGEWPPVIONPEYK/G	3	595.6	0.02	6.29	3.36E-07	208-222	1	.....	.....
								R/FEPSNK/G	2	434.71	0.004	6.25	3.40E-07	81-87	0	.....	.....
								K/IDDPDTSKPDWDKPEHIPDPAK/K	3	920.78	-0.021	6.24	3.03E-07	225-248	0	.....	.....
								K/DNSOVSESSLEDWDFLPPK/K	3	840.82	-0.417	6.19	4.20E-07	186-207	1	.....	.....
								K/HEONIDCGGYK/L	2	738.83	0.001	6.15	6.27E-07	99-111	0	.....	.....Oys CAM:.....
								A/DPAITK/EOFLDGDWNR/W	3	763.45	-0.751	6.03	1.45E-06	18-36	half	.....	.....
								R/KVSELOEEAF/K/K	2	769.18	-1.75	16.5	1.02E-57	222-234	1	.....	.....
								R/VOVSLTCEVDALK/G	2	746.35	-0.47	14.4	4.52E-44	321-333	0	.....	.....Oys CAM:.....
								R/LLODSVDFSLADAINTEK/N	3	709.48	-0.12	14.2	8.63E-43	78-96	0	.....	.....
								R/LLODSVDFSLADAINTEK/N	2	1064.08	-0.544	13.4	9.71E-38	78-96	0	.....	.....
								R/KVSELOEEAF/K/K	2	767.54	-0.11	12.5	5.28E-33	222-234	1	.....	.....
								K/LOEMLQREAEASTLOSFR/O	3	775.74	-0.362	12.4	2.90E-32	188-206	1	.....	.....
								O/DSVDFSLADAINTEK/N	2	886.49	-0.067	12.4	2.77E-32	81-96	half	.....	.....
								K/VSELOEEAF/K/K	2	703.54	-0.157	12	2.75E-30	223-234	0	.....	.....
								R/MEENFALEAANYODITGR/L	2	1101.62	-0.625	11.9	8.58E-30	345-363	0	.....	.....
								R/FEAEASTLOSFR/O	2	648.87	-0.063	11.9	8.79E-30	196-206	0	.....	.....
								K/LLAELOK/G	2	585.72	-0.359	11.8	5.22E-29	129-138	0	.....	.....
								R/MEENFALEAANYODITGR/L	2	1101.58	-0.585	11.6	4.31E-28	345-363	0	.....	.....
								K/FADLSEANR/N	2	547.49	-0.222	11.6	8.85E-28	294-303	0	.....	.....
								R/TKVELOELNDR/F	2	794.47	-0.067	11.3	1.34E-26	100-112	1	.....	.....
								R/LGDLYEEMR/E	2	628	-0.213	11.2	3.28E-26	145-154	0	.....	.....
								R/ISLPLNF/S	1	900.84	-0.321	11.2	1.45E-26	410-417	half	.....	.....
								R/KVSELOEEAF/K/K	2	767.58	-0.15	11.1	1.34E-25	222-234	1	.....	.....
								R/MEENFALEAANYODITGR/L	2	1109.03	-0.038	11.1	7.59E-26	345-363	0	.....	.....Oxidation M:.....
								K/NLOEAEEWY/S	2	655.54	-0.233	11	3.52E-25	282-291	0	.....	.....
								R/LLODSVDFSLADAINTEK/N	2	1063.58	-0.044	10.8	1.25E-24	78-96	0	.....	.....
								K/FADLSEANR/N	2	547.38	-0.112	10.6	5.28E-23	294-303	0	.....	.....
								R/ODVDNASLAR/L	2	544.83	-0.06	10.6	3.24E-23	207-216	0	.....	.....
								R/KVSELOEEAF/K/K	2	767.58	-0.15	10.6	1.91E-23	222-234	1	.....	.....
								R/ROVDOLINDKAR/V	3	482.05	-0.123	10.5	1.26E-22	158-169	2	.....	.....





32	Lamin-A	LMNA	P48679	63.2	7	6	11	R/LSKEDIER/M	2	495.24	0.027	6.26	4.65E-07	510-517	1	.....	.....
								K/OTOTFITITYSDNOPGVLIOWYEGERA	2	1387.71	-0.043	6.25	3.55E-07	424-447	0	.....	.....
								K/LLDDFFNGKE	2	541.28	0.008	6.13	8.24E-07	349-357	0	.....	.....
								K/CNEISWLDK/N	2	638.39	0.924	6.08	1.06E-06	574-583	0	.....	.....
								K/LLDDFFNGKE	2	541.75	-0.062	6.06	1.24E-06	349-357	0	.....	.....
								R/SGAASSTPLSPTR/I	2	680.43	-0.083	13.4	8.21E-38	Dec-25	0	.....	.....
								R/TALINATGEEVAMR/K	2	738.48	-0.1	12.3	1.28E-31	528-541	0	.....	.....
								R/TALINATGEEVAMR/K	2	746.45	-0.073	11.1	1.15E-25	528-541	0	.....	.....
								R/SLETENAGLR/L	2	545.39	-0.109	10.1	3.85E-21	51-60	0	.....	.....
								K/AAYEAELGDAR/K	2	583.39	-0.112	9.89	3.95E-20	79-89	0	.....	.....
35	Bifunctional purine biosynthesis protein PURH	ATIC	O35567	65.3	12	7	19	R/SVGGSGGSGFGDNLVTR/S	2	783.92	-0.041	8.74	1.69E-15	629-645	0	.....	.....
								R/LADALDELRA	2	514.91	-0.119	8.69	3.67E-15	241-249	0	.....	.....
								K/DGQVIGIGAGQQSR/I	2	693.35	0.01	13.5	2.27E-38	438-451	0	.....	.....
								R/GAVDIPAAAFK/H	2	574.37	-0.558	10.2	1.24E-21	255-266	0	.....	.....
								R/DVSELTGPEMLGGR/V	2	812.42	-0.032	9.28	1.35E-17	50-64	0	.....	.....
								R/MSSEFGDVALSDVDVPTAK/I	2	1081.58	-0.087	9.04	1.02E-16	312-331	0	.....	.....
								R/DVSELTGPEMLGGR/V	2	804.41	-0.019	8.91	3.84E-16	50-64	0	.....	.....
								K/INGSTCYLOMDOSYKPDENEVR/I	3	845.34	-0.631	8.57	6.95E-15	359-379	0	.....	.....
								R/MSSEFGDVALSDVDVPTAK/I	2	1082.06	-0.567	8.13	2.69E-13	312-331	0	.....	.....
								R/GAVDIPAAAFK/H	2	573.83	-0.018	7.89	2.96E-12	255-266	0	.....	.....
38	T-complex protein 1 subunit gamma	CCT3	Q6P502	20.1	2	2	4	R/EVSDGVARGYEEALK/I	2	903.51	-0.066	7.47	6.12E-11	336-352	0	.....	.....
								R/NIPEDAAHAR/L	2	609.75	0.025	6.92	4.53E-09	80-90	0	.....	.....
								K/INGSTCYLOMDOSYKPDENEVR/I	3	843.97	0.739	6.8	2.26E-08	359-379	0	.....	.....
								K/DGQVIGIGAGQQSR/I	2	693.91	-0.55	6.11	7.98E-07	438-451	0	.....	.....
								R/TMLDSSLEYK/K	2	640.47	-0.109	10.5	4.37E-23	238-248	0	.....	.....
								R/MALDDVMSTLK/K	2	612.41	-0.106	9.55	1.26E-18	128-138	0	.....	.....
								R/LLDSDVDFSLADAITEFK/N	2	1063.7	-0.164	14.9	1.44E-47	78-96	0	.....	.....
								R/OVOSLTCEVDALK/G	2	746.36	-0.48	14.2	1.39E-42	321-333	0	.....	.....
								R/KVESLOEELFK/K	2	768.84	-1.41	13.5	6.68E-39	222-234	1	.....	.....
								O/DSVDFSLADAITEFK/N	2	886.52	-0.097	13.5	7.62E-39	81-96	half	.....	.....
40	Vimentin	VIM	P31000	314.2	58	29	51	K/FADLSEAANR/N	2	547.42	-0.152	12.6	2.27E-33	294-303	0	.....	.....
								R/TNKEVLOELNDR/F	2	794.66	-0.257	12.2	4.99E-31	100-112	1	.....	.....
								K/VESLOEELFK/K	2	703.89	-0.507	12.1	1.62E-30	223-234	0	.....	.....
								R/GDLYEENR/E	2	627.99	-0.203	11.9	9.08E-30	145-154	0	.....	.....
								K/MALDIATYR/K	2	656.45	-0.115	11.8	2.30E-29	390-400	0	.....	.....
								R/LLDSDVDFSLADAITEFK/N	2	1064.29	-0.754	11.8	2.26E-29	78-96	0	.....	.....
								R/EMEENFALEAANYODITGR/L	2	1101.59	-0.595	11.6	6.80E-28	345-363	0	.....	.....
								R/EMEENFALEAANYODITGR/L	2	1109.34	-0.348	11.5	5.62E-28	345-363	0	.....	.....
								R/KVESLOEELFK/K	2	767.55	-0.12	11.4	2.18E-27	222-234	1	.....	.....
								R/QDVDMASLAR/L	2	544.83	-0.06	11.3	1.38E-26	207-216	0	.....	.....
								K/LLAELEOLK/G	2	585.64	-0.279	11.2	4.06E-26	129-138	0	.....	.....
								K/NLOEAEEWYKSK/F	2	762.96	-0.09	11	5.01E-25	282-293	1	.....	.....
								R/EMEENFALEAANYODITGR/L	2	1109.04	-0.048	10.6	2.68E-23	345-363	0	.....	.....
								R/EEAESTLOSFR/O	2	648.93	-0.123	10.6	4.50E-23	196-206	0	.....	.....
								K/LOEEMLOREAEESTLOSFR/O	3	775.53	-0.152	10.5	6.27E-23	188-206	1	.....	.....
								R/TNKEVLOELNDR/F	2	794.52	-0.117	10.5	7.43E-23	100-112	1	.....	.....
								R/ETNLESPLVDTHSK/R	2	842.08	-0.146	10.4	1.66E-22	424-438	0	.....	.....
								K/MALDIATYR/K	2	656.35	-0.015	10.4	8.96E-22	390-400	0	.....	.....
								R/OVOSLTCEVDALK/G	2	746.48	-0.6	10	8.63E-21	321-333	0	.....	.....
								R/OVOSLTCEVDALK/GTNGESLR/O	3	793.48	-0.42	10	2.47E-20	321-341	1	.....	.....
								K/VESLOEELFK/K	2	767.72	-0.29	9.85	5.95E-20	222-234	1	.....	.....
								K/VELOELNDR/F	2	558.4	-0.112	9.81	1.03E-19	104-112	0	.....	.....
								K/LLAELEOLK/G	2	585.44	-0.079	9.78	1.29E-19	129-138	0	.....	.....
								R/DNLAEIMR/L	1	1076.63	-0.126	9.64	3.47E-19	175-183	0	.....	.....
								R/GDLYEENR/E	2	635.9	-0.115	9.48	2.57E-18	145-154	0	.....	.....
								K/LOEEMLOREAEESTLOSFR/O	3	775.98	-0.602	9.39	1.48E-17	188-206	1	.....	.....
								K/VESLOEELFK/K	2	703.5	-0.117	9.18	4.19E-17	223-234	0	.....	.....
								R/DNLAEIMR/L	2	538.8	-0.044	9.05	1.73E-16	175-183	0	.....	.....
								R/VEVERDNLAEIMR/L	3	563.67	-0.056	9.05	1.75E-16	170-183	1	.....	.....
								K/FADLSEAANRNDAL/R/O	3	593.36	-0.401	9	2.81E-16	294-309	1	.....	.....















66	Trocomycin 1 Alpha chain	TPM1	P04692	277.6	69	28	70	K/ATDAEADVASLR/R	2	730.95	-0.079	15.1	1.75E-48	77-90	1	.....
								R/SKOLEDELVSLOK/K	2	759.1	-0.185	14.6	1.68E-45	36-48	1	.....
								R/LATALOKLEFAEADAESER/G	3	734.78	-0.067	14.1	2.22E-42	106-125	2	.....
								R/SKOLEDELVSLOK/K	2	759.9	-0.085	12.5	6.36E-33	36-48	1	.....
								R/IOLVFEELDR/A	2	622.61	-0.28	11.7	1.77E-28	92-101	0	.....
								K/IVTESDLER/A	2	594.87	-1.032	11.5	1.55E-27	169-178	0	.....
								K/OLEDELVSLOK/K	2	652.43	-1.079	11	2.90E-25	38-48	0	.....
								K/ATDAEADVASLR/R	2	667.01	-0.187	10.9	1.26E-24	78-90	0	.....
								K/OLEDELVSLOK/K	2	651.96	-0.609	10.7	2.73E-23	38-48	0	.....
								K/MEIOEOLK/E	2	567.49	-1.182	10.4	2.37E-22	141-149	0	.....
								R/SKOLEDELVSLOK/K	2	758.97	-0.055	10.2	2.36E-21	36-48	1	.....
								K/IGTEDELKYSALK/D	2	759.49	-0.107	10.2	1.59E-21	52-65	1	.....
								R/IOLVFEELDRAOER/L	3	629.41	-0.741	10.2	2.87E-21	91-105	2	.....
								R/AOKDEEKMEIOEOLK/E	2	980.6	-0.093	10.2	1.39E-21	134-149	2	.....
								K/IVTESDLER/A	2	593.9	-0.062	9.87	5.23E-20	169-178	0	.....
								K/IGTEDELKYSALK/D	3	613.76	-0.109	9.61	7.62E-19	50-65	2	.....
								K/OLEDELVSLOK/K	2	651.42	-0.069	9.57	9.88E-19	38-48	0	.....
								R/IOLVFEELDRAOER/L	3	628.79	-0.121	9.55	1.44E-18	91-105	2	.....
								R/IOLVFEELDRAOER/L	3	628.81	-0.141	9.18	4.61E-17	91-105	2	.....
								K/YSOKEDKYTEEIK/V	3	563.83	-0.225	9.06	1.51E-16	214-226	2	.....
								R/KLVITESDLERAER/A	3	600.97	-0.303	9.04	1.67E-16	168-182	2	.....
								R/AOKDEEKMEIOEOLK/E	3	654.54	-0.533	8.96	7.42E-16	134-149	2	.....
								R/AOKDEEKMEIOEOLK/E	3	654.04	-0.033	8.9	5.89E-16	134-149	2	.....
								R/AOKDEEKMEIOEOLK/E	3	659.83	-0.492	8.83	1.21E-15	134-149	2	.....
								K/YSOKEDKYTEEIK/V	3	563.92	-0.315	8.79	1.75E-15	214-226	2	.....
								R/IOLVFEELDR/A	2	622.38	-0.05	8.78	1.70E-15	92-101	0	.....
								R/KLVITESDLER/A	2	657.97	-0.085	8.7	3.07E-15	168-178	1	.....
								K/CAELEELKVTNNLK/S	2	574.4	-0.095	8.67	4.56E-15	141-149	0	.....
								K/CAELEELKVTNNLK/S	2	946.11	-0.132	8.64	4.07E-15	190-205	1	.....
								K/IVTESDLER/A	2	594.2	-0.362	8.61	7.17E-15	169-178	0	.....
								K/IGTEDELKYSALK/D	2	919.96	0.013	8.49	1.43E-14	50-65	2	.....
								R/AOKDEEKMEIOEOLK/E	2	988.59	-0.086	8.48	1.67E-14	134-149	2	.....
								R/HIAEDADRYEEVAR/K	3	601.39	-0.091	8.26	1.76E-13	153-167	2	.....
								R/IOLVFEELDR/A	3	467.5	-0.244	8.26	3.60E-13	91-101	1	.....
								R/IOLVFEELDR/A	2	700.46	-0.079	8.23	1.58E-13	91-101	1	.....
								R/LATALOKLEAEK/A	2	722.42	-0.013	8.2	2.13E-13	106-118	1	.....
								R/IOLVFEELDRAOER/L	3	629.49	-0.821	8.13	4.56E-13	91-105	2	.....
								K/CAELEELKVTNNLK/S	3	631.07	-0.083	8.11	5.25E-13	190-205	1	.....
								R/LATALOKLEAEK/A	2	722.55	-0.143	8.06	6.75E-13	106-118	1	.....
								R/KLVITESDLERAER/A	3	600.87	-0.203	8.03	1.08E-12	168-182	2	.....
								R/KLVITESDLERAER/A	3	601.05	-0.383	7.99	3.70E-12	168-182	2	.....
								R/KLVITESDLERAER/A	3	601.35	-0.683	7.93	6.33E-12	168-182	2	.....
								K/IVSPKLE	2	401.87	-0.114	7.9	3.71E-12	227-233	1	.....
								R/KLVITESDLER/A	3	438.99	-0.064	7.89	4.01E-12	168-178	1	.....
								R/AOKDEEKMEIOEOLK/E	3	654.09	-0.083	7.88	3.47E-12	134-149	2	.....
								R/KLVITESDLERAER/A	3	600.85	-0.183	7.86	1.03E-11	168-182	2	.....
								R/KLVITESDLERAER/A	3	601.39	-0.723	7.78	2.27E-11	168-182	2	.....
								K/DKENALDR/A	2	537.35	-0.067	7.76	9.24E-12	13-21	1	.....
								K/YSALKDAOKLEAEK/K	3	656.16	-0.483	7.76	7.69E-12	60-76	2	.....
								K/IVTESDLERAER/A	2	836.84	-0.391	7.7	1.00E-11	169-182	1	.....
								R/KLVITESDLERAER/A	3	600.68	-0.013	7.65	2.29E-11	168-182	2	.....
								K/CAELEELKVT	2	560.84	-0.077	7.64	2.26E-11	190-198	0	.....
								R/AELSEGKCAELEELK/T	2	918.08	-0.139	7.52	4.14E-11	183-198	1	.....
								R/KLVITESDLERAER/A	3	600.77	-0.103	7.5	7.21E-11	168-182	2	.....
								R/KLVITESDLERAER/A	3	601.26	-0.593	7.49	1.90E-10	168-182	2	.....
								R/KLVITESDLERAER/A	3	600.77	-0.103	7.39	1.57E-10	168-182	2	.....
								K/MEIOEOLK/E	2	566.4	-0.092	7.19	6.82E-10	141-149	0	.....
								K/HIAEDADRYEEVAR/K	3	601.39	-0.091	7.15	1.03E-09	153-167	2	.....
								K/ATDAEADVASLR/R	2	744.94	-0.066	7.14	7.83E-10	78-91	1	.....
								K/STDLEKVAHAK/E	3	485.75	-0.162	6.99	5.98E-09	252-264	1	.....
								K/IVSPKLE	2	401.86	-0.104	6.81	1.25E-08	227-233	1	.....
								R/AELSEGKCAELEELK/T	3	612.43	-0.134	6.74	1.76E-08	183-198	1	.....





























414	Lamin-A	LMNA	P48679	114.4	13	12	20	K/ILGPNILEVAMP/L	2	751.91	-0.028	8.4	3.82E-14	167-180	half	.....:Oxidation M:..
								K/NLOYDYSK/S	2	607.78	0.026	8.11	4.61E-13	142-151	0	.....:Ovs CAM:.....:Ovs CAM:..
								R/VCENPVLGNK/V	2	758.89	-0.503	7.99	1.03E-12	110-122	0	.....:Oxidation M:..
								K/ILGPNILEVAMP/L	2	751.93	-0.048	7.67	1.39E-11	167-180	half	.....:Oxidation M:..
								-1/AOGEPOVOK/L	2	623.27	-0.453	7.66	1.93E-11	01-Nov	0	.....:ACET nterm:.....
								R/VCENPVLGNK/V	2	758.35	0.037	7.61	2.13E-11	110-122	0	.....:Ovs CAM:.....:Ovs CAM:..
								R/SGAONSTPLSPT/R	2	680.4	-0.053	12.1	1.29E-30	Dec-25	0	.....:.....
								R/SVGGSGGSGFONLVR/S	2	783.94	-0.061	12	2.14E-30	629-645	0	.....:.....
								R/TALINATGEVAMR/K	2	738.43	-0.05	11.8	1.82E-29	528-541	0	.....:.....
								R/TALINATGEVAMR/K	2	746.43	-0.053	11.3	1.46E-26	528-541	0	.....:Oxidation M:..
								R/SLEENAGL/R	2	545.33	-0.049	11	3.58E-25	51-60	0	.....:.....
								R/TITESVSR/E	2	574.88	-0.086	10.8	3.75E-24	63-72	0	.....:.....
								R/MOOLDEYOELLDIK/L	2	555.55	-0.086	9.98	1.20E-20	352-366	0	.....:Oxidation M:.....
								K/AAYEALGDAR/K	2	583.39	-0.112	9.96	2.01E-20	79-89	0	.....:.....
								R/LOLELSK/V	2	415.86	-0.107	8.52	1.83E-14	102-108	0	.....:.....
								R/LADALOELR/A	2	514.91	-0.119	7.8	6.72E-12	241-249	0	.....:.....
								R/LSPTSISOR/S	2	486.78	-0.021	7.64	2.35E-11	389-397	0	.....:.....
								K/NITYSEELR/E	2	512.33	-0.071	6.45	1.10E-07	209-216	0	.....:.....
								K/EGDLAAOAR/L	2	522.33	-0.052	6.23	4.99E-07	124-133	0	.....:.....
									26S protease regulatory subunit 7	PSMC2	Q63347	5.7/6	18	7	19	K/FWDLSDQVAPTDIEEGMR/V
-1/PDYLGDOR/K	2	519.14	-1.391	10.3	2.22E-21	01-Sep	0									.....:.....
R/SVCTEAGMFAIR/A	2	671.26	0.058	9.85	6.08E-20	386-397	0									.....:Ovs CAM:.....
K/FWDLSDQVAPTDIEEGMR/V	2	1061.05	-0.038	9.24	1.50E-17	120-138	0									.....:.....
-1/PDYLGDOR/K	2	517.71	0.039	9.08	1.05E-16	01-Sep	0									.....:.....
R/ALDEGDIALK/T	2	579.35	-0.026	8.67	4.08E-15	23-33	0									.....:.....
R/SVCTEAGMFAIR/A	2	679.34	-0.024	8.66	4.23E-15	386-397	0									.....:Ovs CAM:.....:Oxidation M:.....
K/FWDLSDQVAPTDIEEGMR/V	2	1061.07	-0.058	8.6	5.19E-15	120-138	0									.....:.....
K/ITYGOSTYSR/O	2	532.21	-0.464	7.83	5.26E-12	34-42	0									.....:.....
K/VEDDIOOLLK/K	2	664.88	-0.023	7.76	7.68E-12	46-56	0									.....:.....
								-1/PDYLGDOR/K	2	517.65	0.099	7.69	1.47E-11	01-Sep	0	.....:.....
								-1/PDYLGDOR/K	2	518.22	-0.471	7.5	2.24E-10	01-Sep	0	.....:.....
								K/ITYGOSTYSR/O	2	531.75	-0.004	7.19	7.09E-10	34-42	0	.....:.....
								K/VEDDIOOLLK/K	2	666.14	-1.283	7.12	2.68E-09	46-56	0	.....:.....
								K/FWDLSDQVAPTDIEEGMR/V	2	1069.81	-0.8	6.8	6.56E-09	120-138	0	.....:.....:Oxidation M:..
								K/FWDLSDQVAPTDIEEGMR/V	2	1069.55	-0.54	6.45	1.78E-07	120-138	0	.....:.....:Oxidation M:..
								P/VIGSELVOK/Y	2	486.76	0.03	6.18	6.82E-07	239-247	half	.....:.....
								K/FWDLSDQVAPTDIEEGMR/V	2	1068.81	0.2	6.06	2.18E-06	120-138	0	.....:.....:Oxidation M:..
								R/HTTIFSEGR/L	2	578.81	-0.001	9.07	1.11E-16	Dec-21	0	.....:.....
									Proteasome subunit Alpha type 6	PSMA6	P60901	6.8/7	3	3	13	K/OTESTSLEK/K
R/GKDCAVVTK/K	2	609.79	0.039	8.09	5.86E-13	44-54	1									.....:Ovs CAM:.....
***																



SUPPLEMENTAL TABLE 2 on peptide identification

spot #	Identified protein	symbol	Uniprot-Swiss-Prot #	LC-MS/MS platform	Score	# pept	# valid	# unique	cov%	sequence	charge	m/z	d m/z	z-score	P-value	Pos	#MC	Modif
1	Calretulin	CALR	P18418	LC-ESI-IT	136.6	37	37	16	53%	K/DHMGDSYINMFGDPCGGTK/K	3	814.68	-0.335	13.31	5.21E-38	104-125	0	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/K	2	1196.1	-0.067	12.97	1.99E-36	169-189	0	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/K	2	1196.2	-0.107	12.8	1.72E-35	169-189	0	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/K	2	1196.1	-0.047	12.19	3.52E-32	169-189	0	.....:Cys CAM:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	814.35	-0.005	11.41	9.17E-28	104-125	0	.....:Cys CAM:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	819.08	0.597	11.34	1.64E-27	104-125	0	.....:Cys CAM:.....
										K/EOFLDGDWNTNR/W	2	726.39	-0.059	10.14	7.96E-22	Aug-19	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.52	0.248	9.92	3.44E-21	232-255	0	.....:Oxidation M:.....
										K/SGTIDFNLITNDYAEAEFGNETWGVTK/A	3	1090.3	-0.13	9.6	1.33E-19	306-334	0	.....:Oxidation M:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	820.09	-0.413	9.51	9.78E-19	104-125	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	987.81	-0.374	9.4	6.83E-19	232-255	0	.....:Cys CAM:.....
										K/FYGDQEKGLQTSODAR/F	2	1043.7	-0.155	9.3	5.32E-18	39-56	2	.....:Cys CAM:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	987.83	-0.394	8.42	4.80E-15	232-255	0	.....:Cys CAM:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	825.04	-0.032	8.41	6.79E-15	104-125	0	.....:Oxidation M:.....
										K/KDPDAKPEDWDER/A	3	595.64	-0.02	8.35	1.74E-14	191-205	1	.....:Cys CAM:.....
										K/NVLINK/D	1	700.46	-0.025	8.28	1.90E-14	137-142	0	.....:Cys CAM:.....
										K/HEONIDCGGYK/L	2	738.88	-0.05	7.96	4.41E-13	82-94	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.82	-0.052	7.86	3.81E-13	232-255	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.81	-0.042	7.69	1.52E-12	232-255	0	.....:Oxidation M:.....
										K/SGTIDFNLITNDYAEAEFGNETWGVTK/A	2	1636.3	-1.559	7.64	2.04E-12	306-334	0	.....:Cys CAM:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	819.66	0.017	7.6	5.63E-12	104-125	0	.....:Oxidation M:.....
										K/EOFLDGDWNTNR/W	2	726.32	0.011	7.51	1.18E-11	Aug-19	0	.....:Cys CAM:.....
										K/FYGDQEKGLQTSODAR/F	3	696	-0.001	7.19	1.40E-10	39-56	2	.....:Cys CAM:.....
										K/PEPYNK/G	2	434.71	0.004	7.05	3.49E-10	64-70	0	.....:Cys CAM:.....
										-/DPATYK/E	2	427.24	-0.014	6.9	1.09E-09	01-Jul	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.17	0.598	6.88	6.93E-10	232-255	0	.....:Oxidation M:.....
										K/DHMGDSYINMFGDPCGGTK/K	3	820.05	-0.373	6.79	2.07E-09	104-125	0	.....:Cys CAM:.....
										K/HEONIDCGGYK/L	2	738.83	0	6.78	2.78E-09	82-94	0	.....:Cys CAM:.....
										-/DPATYK/EOFLDGDWNTNR/W	3	763.45	-0.751	6.73	3.56E-09	Jan-19	1	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/I	3	840.82	-0.417	6.72	3.51E-09	169-190	1	.....:Cys CAM:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	988.14	-0.704	6.58	2.11E-08	232-255	0	.....:Cys CAM:.....
										-/DPATYK/E	1	852.97	0.475	6.56	6.61E-09	01-Jul	0	.....:Cys CAM:.....
										R/FYALSAR/F	2	414.18	0.044	6.55	1.12E-08	57-63	0	.....:Cys CAM:.....
										K/FVLSSGK/F	1	737.44	-0.021	6.48	1.06E-08	32-38	0	.....:Cys CAM:.....
										K/IDDPDTSKPEDWKPKEHIPDPDAK/K	3	920.78	-0.021	6.36	3.17E-08	208-231	0	.....:Cys CAM:.....
										K/KDPDAKPEDWDER/A	3	595.6	0.02	6.3	7.83E-08	191-205	1	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/I	3	840.43	-0.027	6.11	1.81E-07	169-190	1	.....:Cys CAM:.....
										K/DNSOVESGSLDDWDFLPKK/I	3	840.39	0.01	12.59	4.73E-34	169-190	1	.....:Cys CAM:.....
1	Calretulin	CALR	P18418	LC-ESI-Q-TOF	45.4	5	5	5	21%									
73	Elongation factor 2	EEF2	P05197	LC-ESI-IT	51.2	11	11	5	8%	R/CKDDEFTHLYTLVRPNTYEVK/I	4	714.85	0.005	9.97	5.29E-21	146-168	1	.....:Cys CAM:.....
										K/EOFLDGDWNTNR/W	2	726.33	0.001	8.79	3.85E-16	Aug-19	0	.....:Cys CAM:.....
										ANVLGLDLWQV/KS	2	585.86	-0.009	7.21	1.41E-10	296-305	half	.....:Cys CAM:.....
										K/KDPDAKPEDWDER/A	3	595.62	-0.002	6.88	1.86E-09	191-205	1	.....:Cys CAM:.....
										R/FETYSSESNVLSLK/S	2	797.89	-0.005	12.56	6.98E-34	580-593	0	.....:Cys CAM:.....
										R/CELLYEGPPDDEAAMGK/S	2	1012	0.453	12.28	1.77E-32	368-385	0	.....:Oxidation M:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.07	-0.039	11.43	7.05E-28	605-624	1	.....:Cys CAM:.....
										R/CELLYEGPPDDEAAMGK/S	2	1004.5	-0.044	10.59	6.38E-24	368-385	0	.....:Cys CAM:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.08	-0.049	9.98	4.11E-21	605-624	1	.....:Cys CAM:.....
										R/FETYSSESNVLSLK/S	2	797.92	-0.035	9.82	1.94E-20	580-593	0	.....:Cys CAM:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.33	-0.299	9.33	5.92E-18	605-624	1	.....:Cys CAM:.....
										R/CELLYEGPPDDEAAMGK/S	2	1012.5	-0.057	7.63	3.86E-12	368-385	0	.....:Cys CAM:.....
										R/CELLYEGPPDDEAAMGK/S	2	484.18	1.097	7.47	1.69E-11	716-725	0	.....:Oxidation M:.....
										-/NNTFYDQIR/A	2	546.79	-0.494	7.43	2.63E-11	01-Sep	0	.....:Cys CAM:.....
										R/GGGQIIPTR/R	2	485.28	-0.003	7.34	4.21E-11	716-725	0	.....:Cys CAM:.....



73	Elongation factor 2	EEF2	P05197	LC-ESI-Q-TOF	32.0	4	4	4	4	6%	K/ARPPDGLAEDIDKGEYSAR/Q	3	715.02	0.01	9.93	6.41E-21	605-624	1	.....	.....
											RVFSGWSTGLK/V	2	546.32	1.001	7.53	9.54E-12	415-425	0	.....	.....
											K/SLTIDSLVCK/A	2	562.28	0.003	7.45	1.77E-11	32-41	0	.....	.....
											K/GEGLGAER/A	2	494.24	0.008	7.12	2.71E-10	239-248	0	.....	.....
92	Elongation factor 2	EEF2	P05197	LC-ESI-IT	19.9	3	3	2	2	4%	R/VTFSESNWLCK/S	2	797.79	0.095	10.28	1.75E-22	580-593	0	.....	.....
											R/CELLYEGPDDDEAMGK/S	2	1012.4	0.083	9.6	1.57E-19	368-385	0	.....	.....
											R/CELLYEGPDDDEAMGK/S	2	1004.4	0.076	8.35	1.01E-14	368-385	0	.....	.....
92	Elongation factor 2	EEF2	P05197	LC-ESI-Q-TOF																
95	Vinculin *	VCL	gi114903	LC-ESI-IT	478.6	128	128	49	48%		K/AQQVSGQLDVLTA/V	2	729.44	-0.038	15.13	1.78E-48	353-366	0	.....	.....
			1251								R/SLGEIAALTSK/L	2	545.37	-0.059	13.93	9.85E-41	434-444	0	.....	.....
											R/SLGEIAALTSK/L	2	545.28	0.031	13.6	8.62E-39	434-444	0	.....	.....
											K/MTGLVDEADTK/S	2	654.83	-0.009	13.31	3.42E-37	709-720	0	.....	.....
											K/TOMOEAMTOESDVFSDTTPIK/L	2	1302.2	-0.094	13.3	2.10E-37	585-607	0	.....	.....
											K/TOMOEAMTOESDVFSDTTPIK/L	2	1311.2	-1.066	13.19	8.58E-37	585-607	0	.....	.....
											K/MTGLVDEADTK/S	2	646.85	-0.026	13.07	8.35E-36	709-720	0	.....	.....
											R/VLOLTSWDEDAWASK/D	2	875	-0.072	12.96	3.09E-35	247-261	0	.....	.....
											K/MLGOMTDVADLR/A	2	755.4	-0.045	12.68	1.29E-33	327-339	0	.....	.....
											K/OVATALONLOT/K/T	2	657.9	-0.028	12.59	1.19E-32	465-476	0	.....	.....
											R/MALLMAEMSR/L	2	576.82	-0.038	12.53	9.66E-33	926-935	0	.....	.....
											K/MSAEINEIR/V	2	588.37	-0.062	12.08	2.79E-30	237-246	0	.....	.....
											R/TNIDSESEQATEMLVH/N	2	974.95	-0.024	11.86	2.41E-29	1009-1025	half	.....	.....
											K/MTGLVDEADTK/S	2	646.81	0.014	11.79	7.80E-29	709-720	0	.....	.....
											R/TDAGFTLR/W	2	440.73	0	11.65	4.62E-28	1050-1057	0	.....	.....
											K/MLGOMTDVADLR/A	2	747.4	-0.042	11.51	1.80E-27	327-339	0	.....	.....
											K/AQQVSGQLDVLTA/V	2	730.24	-0.838	11.35	1.33E-26	353-366	0	.....	.....
											K/STVEGIOASV/K/T	2	559.84	-0.034	11.33	2.08E-26	656-666	0	.....	.....
											K/AVAGNISDPGLK/S	2	635.35	-0.006	11.32	1.83E-26	803-815	0	.....	.....
											K/TOMOEAMTOESDVFSDTTPIK/L	2	1302.7	-0.564	11.31	1.07E-26	585-607	0	.....	.....
											R/VKGETVOTTEIDILK/R	2	844.97	-0.013	11.17	8.66E-26	57-71	1	.....	.....
											R/VLOLTSWDEDAWASK/D	2	875.46	-0.532	11.17	8.53E-26	247-261	0	.....	.....
											R/MALLMAEMSR/L	2	584.82	-0.04	11.06	3.60E-25	926-935	0	.....	.....
											K/MSAEINEIR/V	2	596.25	0.056	10.8	6.81E-24	237-246	0	.....	.....
											R/ALASOLOSLK/D	2	587.33	-0.003	10.75	1.36E-23	571-581	0	.....	.....
											K/TOMOEAMTOESDVFSDTTPIK/L	2	1310.1	-0.046	10.72	7.13E-24	585-607	0	.....	.....
											R/EAFOPDPFPFPPDLEQLR/L	2	1224.7	-0.572	10.68	1.21E-23	833-853	0	.....	.....
											R/MALLMAEMSR/L	2	584.85	-0.07	10.58	6.81E-23	926-935	0	.....	.....
											K/GNDIIAAK/R	2	436.77	-0.025	10.52	1.72E-22	916-924	0	.....	.....
											R/EAFOPDPFPFPPDLEQLR/L	3	817.09	-0.689	10.5	1.24E-22	833-853	0	.....	.....
											K/MSAEINEIR/V	2	596.33	-0.024	10.48	2.22E-22	237-246	0	.....	.....
											K/SLDASEAKK/D	2	652.38	-0.021	10.35	7.67E-22	721-732	1	.....	.....
											R/TDAGFTLR/W	2	441.28	-0.55	10.32	1.07E-21	1050-1057	0	.....	.....
											R/VMLVNSMTVK/E	2	634.34	-0.017	10.27	1.60E-21	189-199	0	.....	.....
											R/EAFOPDPFPFPPDLEQLR/L	3	816.45	-0.049	10.26	1.56E-21	833-853	0	.....	.....
											K/MTGLVDEADTK/S	2	653.53	1.291	10.23	6.69E-21	709-720	0	.....	.....
											K/MLGOMTDVADLR/A	2	747.34	0.018	10.19	3.63E-21	327-339	0	.....	.....
											R/ELTPVISAAR/I	2	592.88	-0.045	10.13	7.67E-21	670-680	0	.....	.....
											K/OVATALONLOT/K/T	2	655.9	1.972	10.1	2.45E-20	465-476	0	.....	.....
											R/MALLMAEMSR/L	2	600.81	-0.035	10.05	1.80E-20	926-935	0	.....	.....
											R/ALASOLOSLK/D	2	586.7	0.627	10.02	2.47E-20	571-581	0	.....	.....
											R/MALLMAEMSR/L	2	584.84	-0.06	9.97	9.87E-20	926-935	0	.....	.....
											K/GNDIIAAK/R	2	437.26	-0.515	9.97	5.66E-20	916-924	0	.....	.....
											R/GOGASPVAMOK/A	2	537.28	-0.006	9.84	1.74E-19	342-352	0	.....	.....
											K/MLGOMTDVADLR/A	2	747.24	0.118	9.77	6.95E-19	327-339	0	.....	.....
											R/ALASOLOSLK/D	2	587.29	0.037	9.72	1.39E-18	571-581	0	.....	.....
											K/ELLPVLSAMK/I	2	615.42	-0.058	9.69	6.26E-19	200-210	0	.....	.....
											R/DPNASPGDAGEAIR/Q	2	749.39	-0.04	9.64	8.29E-19	286-300	0	.....	.....
											R/WIDNPTVDRI/G	2	615.82	-0.029	9.59	1.67E-18	503-512	0	.....	.....
											R/LANVMMGPR/R/O	2	592.31	-0.026	9.58	1.75E-18	529-538	0	.....	.....
											R/VMLVNSMTVK/E	2	634.34	-0.017	9.51	3.19E-18	189-199	0	.....	.....























SUPPLEMENTAL TABLE 2 on peptide identification

spot #	Identified protein	symbol	Uniprot-Swiss-Prot #	LC-MS/MS platform	Score	# pept	# valid	# unique	cov%	sequence	charge	m/z	d m/z	z-score	p-value	Pos	#MC	Modif
1	Calreticulin	CALR	P18418	LC-ESI-IT	136.6	37	37	16	53%	K/DHMGDSEYINMGPDICGGTK/K	3	814.68	-0.335	13.31	5.21E-38	104-125	0	.....:Oys CAM:.....
										K/DNSOVESGSLDDWDFLPK/K	2	1196.1	-0.067	12.97	1.99E-36	169-189	0	.....
										K/DNSOVESGSLDDWDFLPK/K	2	1196.2	-0.107	12.8	1.72E-35	169-189	0	.....
										K/DNSOVESGSLDDWDFLPK/K	2	1196.1	-0.047	12.19	3.52E-32	169-189	0	.....
										K/DHMGDSEYINMGPDICGGTK/K	3	814.35	-0.005	11.41	9.17E-28	104-125	0	.....:Oys CAM:.....
										K/DHMGDSEYINMGPDICGGTK/K	3	819.08	0.597	11.34	1.64E-27	104-125	0	.....:Oxidation M:.....:Oys CAM:.....
										K/EOFLDGDWNTNR/W	2	726.39	-0.059	10.14	7.96E-22	Aug-19	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.52	0.248	9.92	3.44E-21	232-255	0	.....:Oxidation M:.....
										K/SGTIDFNFLITNDEAYAEFGNETWGVTK/A	3	1090.3	-0.13	9.6	1.33E-19	306-334	0	.....:Oxidation M:.....
										K/DHMGDSEYINMGPDICGGTK/K	3	820.09	-0.413	9.51	9.78E-19	104-125	0	.....:Oxidation M:.....:Oys CAM:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	987.81	-0.374	9.4	6.83E-19	232-255	0	.....
										K/FYGDQEKGLGLOTSDAR/F	2	1043.7	-0.155	9.3	5.32E-18	39-56	2	.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	987.83	-0.394	8.42	4.80E-15	232-255	0	.....
										K/DHMGDSEYINMGPDICGGTK/K	3	825.04	-0.032	8.41	6.79E-15	104-125	0	.....:Oxidation M:.....:Oxidation M:.....:Oys CAM:.....
										K/KIPDPAAKPEDWDER/A	3	595.64	-0.02	8.35	1.74E-14	191-205	1	.....
										K/NVLINK/D	1	700.46	-0.025	8.28	1.90E-14	137-142	0	.....
										K/HEONIDCGGYK/L	2	738.88	-0.05	7.96	4.41E-13	82-94	0	.....:Oys CAM:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.82	-0.052	7.86	3.81E-13	232-255	0	.....:Oxidation M:.....
										K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.81	-0.042	7.69	1.52E-12	232-255	0	.....:Oxidation M:.....
										K/SGTIDFNFLITNDEAYAEFGNETWGVTK/A	2	1636.3	-1.559	7.64	2.04E-12	306-334	0	.....
K/DHMGDSEYINMGPDICGGTK/K	3	819.66	0.017	7.6	5.63E-12	104-125	0	.....:Oxidation M:.....:Oys CAM:.....										
K/EOFLDGDWNTNR/W	2	726.32	0.011	7.51	1.18E-11	Aug-19	0	.....										
K/FYGDQEKGLGLOTSDAR/F	3	696	-0.001	7.19	1.40E-10	39-56	2	.....										
R/FEPPSNK/G	2	434.71	0.004	7.05	3.49E-10	64-70	0	.....										
-/DPAITYK/E	2	427.24	-0.014	6.9	1.09E-09	01-Jul	0	.....										
K/KPEDWDEMDGEWPPVIONPEYK/G	3	992.17	0.598	6.88	6.93E-10	232-255	0	.....:Oxidation M:.....										
K/DHMGDSEYINMGPDICGGTK/K	3	820.05	-0.373	6.79	2.07E-09	104-125	0	.....:Oxidation M:.....:Oys CAM:.....										
K/HEONIDCGGYK/L	2	738.83	0	6.78	2.78E-09	82-94	0	.....:Oys CAM:.....										
-/DPAITYK/EOFLDGDWNTNR/W	3	763.45	-0.751	6.73	3.56E-09	Jan-19	1	.....										
K/DNSOVESGSLDDWDFLPKK/I	3	840.82	-0.417	6.72	3.51E-09	169-190	1	.....										
K/KPEDWDEMDGEWPPVIONPEYK/G	3	988.14	-0.704	6.58	2.11E-08	232-255	0	.....										
-/DPAITYK/E	1	852.97	0.475	6.56	6.61E-09	01-Jul	0	.....										
R/FYALSAR/F	2	414.18	0.044	6.55	1.12E-08	57-63	0	.....										
K/FVLSSGK/F	1	737.44	-0.021	6.48	1.06E-08	32-38	0	.....										
K/IDDDTDSKPEDWDKPEHIPDPDAK/K	3	920.78	-0.021	6.36	3.17E-08	208-231	0	.....										
K/KIPDPAAKPEDWDER/A	3	595.6	0.02	6.3	7.83E-08	191-205	1	.....										
K/DNSOVESGSLDDWDFLPKK/I	3	840.43	-0.027	6.11	1.81E-07	169-190	1	.....										
K/DNSOVESGSLDDWDFLPKK/I	3	840.39	0.01	12.59	4.73E-34	169-190	1	.....										
1	Calreticulin	CALR	P18418	LC-ESI-Q-TOF	45.4	5	5	5	21%	R/CKDDPEFTHLYLVRPNTIYEVK/I	4	714.85	0.005	9.97	5.29E-21	146-168	1	.....:Oys CAM:.....
										K/EOFLDGDWNTNR/W	2	726.33	0.001	8.79	3.85E-16	Aug-19	0	.....
										ANVLGLDWOK/S	2	585.86	-0.009	7.21	1.41E-10	296-305	half	.....
										K/KIPDPAAKPEDWDER/A	3	595.62	-0.002	6.88	1.86E-09	191-205	1	.....
										R/ETVSEESNVLCLSK/S	2	797.89	-0.005	12.56	6.98E-34	590-593	0	.....:Oys CAM:.....
										R/CELLYEGPPDDEAAMGK/S	2	1012	0.453	12.28	1.77E-32	368-385	0	.....:Oys CAM:.....:Oxidation M:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.07	-0.039	11.43	7.05E-28	605-624	1	.....
										R/CELLYEGPPDDEAAMGK/S	2	1004.5	-0.044	10.59	6.38E-24	368-385	0	.....:Oys CAM:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.08	-0.049	9.98	4.11E-21	605-624	1	.....
										R/ETVSEESNVLCLSK/S	2	797.92	-0.035	9.82	1.94E-20	580-593	0	.....:Oys CAM:.....
										K/ARPPDGLAEDIDKGVSAK/O	3	715.33	-0.299	9.33	5.92E-18	605-624	1	.....
										R/CELLYEGPPDDEAAMGK/S	2	1012.5	-0.057	7.63	3.86E-12	368-385	0	.....:Oys CAM:.....:Oxidation M:.....
										R/GGGQIIPTR/R	2	484.18	1.097	7.47	1.69E-11	716-725	0	.....
										-/NFTVDQIR/A	2	546.79	-0.494	7.43	2.63E-11	01-Sep	0	.....
										R/GGGQIIPTR/R	2	485.28	-0.003	7.34	4.21E-11	716-725	0	.....



73	Elongation factor 2	EEF2	P05197	LC-ESI-Q-TOF	32.0	4	4	4	6%	K/ARPPDGLAEDIDKGEVSAR/Q	3	715.02	0.01	9.93	6.41E-21	605-624	1	.....
										R/VFSGWSTGLK/V	2	546.32	1.001	7.53	9.54E-12	415-425	0	.....
										K/STLDSLVCK/A	2	562.28	0.003	7.45	1.77E-11	32-41	0	.....:Cys CAM:..
										K/GEGLGAER/A	2	494.24	0.008	7.12	2.71E-10	239-248	0	.....
92	Elongation factor 2	EEF2	P05197	LC-ESI-IT	19.9	3	3	2	4%	R/VTSVESNLGLSK/S	2	797.79	0.095	10.28	1.75E-22	580-593	0	.....:Cys CAM:..
										R/CYLYEGPDDAEAMGK/S	2	1012.4	0.083	9.6	1.57E-19	368-385	0	.....:Cys CAM:..
										R/CELLYEGPDDAEAMGK/S	2	1004.4	0.076	8.35	1.01E-14	368-385	0	.....:Cys CAM:..
92	Elongation factor 2	EEF2	P05197	LC-ESI-Q-TOF														.....
95	Vinculin *	VCL	g014903	LC-ESI-IT	478.6	128	128	49	48%	K/AQQVSGQLDVLTK/V	2	729.44	-0.038	15.13	1.78E-48	353-366	0	.....
			1251							R/SLGEAALT/SK/L	2	545.37	-0.059	13.93	9.85E-41	434-444	0	.....
										R/SLGEAALT/SK/L	2	545.28	0.031	13.6	8.62E-39	434-444	0	.....
										K/MTGLVDEADTK/S	2	654.83	-0.009	13.31	3.42E-37	709-720	0	.....:Oxidation M:.....
										K/TMOEAMTQSDVFSDDTTPIK/L	2	1302.2	-0.094	13.3	2.10E-37	585-607	0	.....:Oxidation M:.....
										K/TMOEAMTQSDVFSDDTTPIK/L	2	1311.2	-0.066	13.19	8.56E-37	585-607	0	.....:Oxidation M:.....
										K/MTGLVDEADTK/S	2	646.85	-0.026	13.07	8.35E-36	709-720	0	.....
										R/VLOLTSWDEDAWASK/D	2	875	-0.072	12.96	3.09E-35	247-261	0	.....
										K/MLGOMTDQVADLR/A	2	755.4	-0.045	12.68	1.29E-33	327-339	0	.....:Oxidation M:.....
										K/OVATALONLOT/K/T	2	657.9	-0.028	12.59	1.19E-32	465-476	0	.....
										R/MALLMAEMSR/L	2	576.82	-0.038	12.53	9.66E-33	926-935	0	.....
										K/MSAEINEIR/V	2	588.37	-0.062	12.08	2.79E-30	237-246	0	.....
										R/TNISDEESEQATEMLVH/N	2	974.95	-0.024	11.86	2.41E-29	1009-1025	half	.....:Oxidation M:.....
										K/MTGLVDEADTK/S	2	648.81	0.014	11.79	7.80E-29	709-720	0	.....
										R/TDAGFTLR/W	2	440.73	0	11.65	4.62E-28	1050-1057	0	.....
										K/MLGOMTDQVADLR/A	2	747.4	-0.042	11.51	1.80E-27	327-339	0	.....:Oxidation M:.....
										K/AQVSGQLDVLTK/V	2	730.24	-0.838	11.35	1.33E-26	353-366	0	.....
										K/STVEGLOSK/V/T	2	559.84	-0.034	11.33	2.08E-26	656-666	0	.....
										K/AVAGNISDPGLQK/S	2	635.35	-0.006	11.32	1.83E-26	803-815	0	.....
										K/TMOEAMTQSDVFSDDTTPIK/L	2	1302.7	-0.564	11.31	1.07E-26	585-607	0	.....:Oxidation M:.....
										R/VKGTVOITTEDOLK/R	2	844.97	-0.013	11.17	8.66E-26	57-71	1	.....
										R/VLOLTSWDEDAWASK/D	2	875.46	-0.532	11.17	8.53E-26	247-261	0	.....
										R/MALLMAEMSR/L	2	584.82	-0.04	11.06	3.60E-25	926-935	0	.....:Oxidation M:.....
										K/MSAEINEIR/V	2	596.25	0.056	10.8	6.81E-24	237-246	0	.....:Oxidation M:.....
										R/ALASLODSLK/D	2	587.33	-0.003	10.75	1.36E-23	571-581	0	.....
										K/TMOEAMTQSDVFSDDTTPIK/L	2	1310.1	-0.046	10.72	7.13E-24	585-607	0	.....:Oxidation M:.....
										R/EAFQPEPDPFPPPPDLEQL/R/L	2	1224.7	-0.572	10.68	1.21E-23	833-853	0	.....
										R/MALLMAEMSR/L	2	584.85	-0.07	10.58	6.81E-23	926-935	0	.....:Oxidation M:.....
										K/GNDITAAK/R	2	436.77	-0.025	10.52	1.72E-22	916-924	0	.....
										R/EAFQPEPDPFPPPPDLEQL/R/L	3	817.09	-0.689	10.5	1.24E-22	833-853	0	.....
										K/MSAEINEIR/V	2	596.33	-0.024	10.48	2.22E-22	237-246	0	.....:Oxidation M:.....
										K/SLDASEEATK/D	2	652.38	-0.021	10.35	7.67E-22	721-732	1	.....
										R/TDAGFTLR/W	2	441.28	-0.55	10.32	1.07E-21	1050-1057	0	.....
										R/VMLVNSMNTVK/E	2	634.34	-0.017	10.27	1.60E-21	189-199	0	.....:Oxidation M:.....
										R/EAFQPEPDPFPPPPDLEQL/R/L	3	816.45	-0.049	10.26	1.56E-21	833-853	0	.....
										K/MTGLVDEADTK/S	2	653.53	1.291	10.23	6.69E-21	709-720	0	.....:Oxidation M:.....
										K/MLGOMTDQVADLR/A	2	747.34	0.018	10.19	3.63E-21	327-339	0	.....:Oxidation M:.....
										R/ELTPQVISAAR/L	2	592.88	-0.045	10.13	7.67E-21	670-680	0	.....
										K/OVATALONLOT/K/T	2	655.9	1.972	10.1	2.45E-20	465-476	0	.....
										R/MALLMAEMSR/L	2	600.81	-0.035	10.05	1.80E-20	926-935	0	.....:Oxidation M:.....
										R/ALASLODSLK/D	2	586.7	0.627	10.02	2.47E-20	571-581	0	.....
										R/MALLMAEMSR/L	2	584.84	-0.06	9.97	9.87E-20	926-935	0	.....:Oxidation M:.....
										K/GNDITAAK/R	2	437.26	-0.515	9.97	5.66E-20	916-924	0	.....
										R/GOGASPVAMQK/A	2	537.28	-0.006	9.84	1.74E-19	342-352	0	.....
										K/MLGOMTDQVADLR/A	2	747.24	0.118	9.77	6.95E-19	327-339	0	.....:Oxidation M:.....
										R/ALASLODSLK/D	2	587.29	0.037	9.72	1.39E-18	571-581	0	.....
										K/ELPLYSAMK/I	2	615.42	-0.058	9.69	6.26E-19	200-210	0	.....:Oxidation M:..
										R/DPNAPSGDAGEAIR/O	2	749.39	-0.04	9.64	8.29E-19	286-300	0	.....
										R/MIDNPTVDPR/G	2	615.82	-0.029	9.59	1.67E-18	503-512	0	.....
										R/LANVMGPHY/O	2	592.31	-0.026	9.58	1.75E-18	529-538	0	.....:Oxidation M:.....
										R/VMLVNSMNTVK/E	2	634.34	-0.017	9.51	3.19E-18	189-199	0	.....:Oxidation M:.....



95	Vinculin	VCL	g114903	LC-ESI-Q-TOF	33.3	4	4	4	4%	R/NTSDSESOATEMLVH/N	2	975.43	-0.504	6.53	8.19E-08	1009-1025	half	.....Oxidation M:.....
										R/EAFQPOEPFPPPPDLEOLR/L	3	817.41	-1.009	6.51	3.41E-07	833-853	0	.....
										R/NFTVGK/M	1	665.44	-0.078	6.47	1.09E-07	231-236	0	.....
										K/AGEVINOPMMAAR/Q	2	776.36	-0.5	6.47	1.63E-07	890-903	0	.....Oxidation M:.....
										L/DASEEAK/D	2	550.73	1.571	6.41	7.89E-07	723-732	half	.....
										R/ALASOLOSLK/D	2	587.81	-0.483	6.4	3.43E-07	571-581	0	.....
										K/OVATALONLOT/K	2	658.4	-0.528	6.33	4.51E-07	465-476	0	.....
										K/TISPMVMDAK/A	2	562.81	-0.04	6.25	7.36E-07	793-802	0	.....Oxidation M:.....
										K/NSKNGIEEAL/K	2	665.7	0.152	6.22	8.97E-07	217-228	1	.....
										R/ELTPOVISAAR/L	2	593.35	-0.515	6.22	1.03E-06	670-680	0	.....
										R/OILDGAKNGELCAGK/E	3	563.3	-0.006	6.18	1.42E-06	301-316	1	.....Ovs CAM:.....
										K/AAVGAANKSTVEGOASV/K	3	624.89	-0.212	6.17	1.33E-06	647-666	1	.....
										R/EAFQPOEPFPPPPDLEOLR/L	2	1225.2	-1.082	6.09	1.13E-06	833-853	0	.....
										R/WIDNPTVDNR/G	2	616.39	-0.599	6.06	2.69E-06	503-512	0	.....
										K/TISPMVMDAK/A	2	554.76	0.012	6.04	7.69E-06	793-802	0	.....Oxidation M:.....
										R/DTAGFTLR/W	2	440.73	-0.003	9.27	3.45E-17	1050-1057	0	.....
										K/VAGNISDPGLOK/S	2	634.32	1.023	9.26	3.50E-17	803-815	0	.....
										R/DPNASPGDAGEAIR/O	2	749.85	-0.502	7.41	1.84E-10	286-300	0	.....
										R/PTISTOLK/I	2	500.81	-0.009	7.35	4.37E-10	988-996	0	.....
125	Vimentin	VM	P31000	LC-ESI-IT	642.2	175	175	62	72%	R/LOOSVDFSLADAINTEK/N	2	1064.2	-0.654	12.14	2.18E-52	75-97	0	.....
										R/LOOSVDFSLADAINTEK/N	2	1063.7	-0.124	15.7	1.62E-52	79-97	0	.....
										R/LOOSVDFSLADAINTEK/N	2	1063.7	-0.134	15.58	9.78E-52	79-97	0	.....
										R/LOOSVDFSLADAINTEK/N	2	1063.7	-0.114	15.34	4.51E-50	79-97	0	.....
										K/VESLOEELAK/K	2	704.39	-1.008	14.57	6.59E-45	224-235	0	.....
										R/LOOSVDFSLADAINTEK/N	2	1063.8	-0.304	14.13	2.70E-42	79-97	0	.....
										R/LOOSVDFSLADAINTEK/N	3	709.62	-0.26	14.03	1.53E-41	79-97	0	.....
										K/MALDIEIATY/K	2	656.86	-0.526	14	6.41E-41	391-401	0	.....Oxidation M:.....
										R/OVOSLTCEVDAL/K	2	746.29	-0.41	13.97	3.52E-41	322-334	0	.....Ovs CAM:.....
										R/LOOSVDFSLADAINTEK/N	2	1064.3	-0.724	13.93	1.13E-40	79-97	0	.....
										R/OVOSLTCEVDAL/KGTNESLERO	3	845.22	-0.126	13.88	9.65E-41	321-342	2	.....Ovs CAM:.....
										R/LOOSVDFSLADAINTEK/N	3	709.35	0.01	13.71	1.37E-39	79-97	0	.....
										R/VESLOEELAK/K	2	745.97	-0.09	13.31	3.16E-37	322-334	1	.....Ovs CAM:.....
										R/OVOSLTCEVDAL/K	2	768.72	-1.29	13.45	1.11E-37	223-235	1	.....
										K/MALDIEIATY/K	2	656.39	-0.056	13.2	1.43E-36	391-401	0	.....Oxidation M:.....
										R/MEENFALEAANYODITGR/L	2	1109.1	-0.138	12.93	2.80E-35	346-364	0	.....Oxidation M:.....
										R/VESLOEELAK/K	2	767.54	-0.11	12.83	1.69E-34	223-235	1	.....
										R/MEENFALEAANYODITGR/L	2	1101	-0.036	12.75	2.96E-34	346-364	0	.....
										K/SRLGDLYEENR/E	2	749.45	-0.096	12.71	7.42E-34	144-155	1	.....
										R/MEENFALEAANYODITGR/L	2	1101.6	-0.636	12.69	1.93E-33	346-364	0	.....
										K/SRLGDLYEENR/E	2	749.46	-0.106	12.58	3.69E-33	144-155	1	.....
										K/INLOFAEYK/S	2	655.61	-0.303	12.53	7.86E-33	283-292	0	.....
										R/ETNIESLPVDTH/S	2	734.43	-0.06	12.42	2.87E-32	425-437	half	.....
										R/VESLOEELAK/K	2	831.65	-0.173	12.42	2.65E-32	223-236	2	.....
										R/MEVLOELNDR/F	2	767.51	-0.08	12.29	1.47E-31	223-235	1	.....
										K/SKADISAAAR/N	2	794.45	-0.048	12.1	1.56E-30	101-113	1	.....
										R/MEVLOELNDR/F	2	654.92	-0.089	12.08	2.21E-30	293-304	1	.....
										K/INLOFAEYK/S	2	794.48	-0.078	11.91	1.56E-29	101-113	1	.....
										R/MEVLOELNDR/F	2	762.96	-0.09	11.89	1.87E-29	283-294	1	.....
										K/FADLSAAR/N	2	547.41	-0.143	11.86	3.24E-29	295-304	0	.....
										R/ONRENEFALEAANYODITGR/L	3	883.41	-0.015	11.84	3.24E-29	343-364	1	.....Oxidation M:.....
										K/INLOFAEYK/S	2	655.35	-0.043	11.76	1.01E-28	283-292	0	.....
										R/MEVLOELNDR/F	2	794.99	-0.588	11.73	1.25E-28	101-113	1	.....
										K/VTETRDGVINETSQHDDLE/-	3	808.47	-0.096	11.72	1.40E-28	446-466	1	.....
										R/VESLOEELAK/K	2	767.73	-0.3	11.67	2.58E-28	223-235	1	.....
										K/SRLGDLYEENR/E	2	749.47	-0.116	11.64	3.48E-28	144-155	1	.....
										K/ARVEVERDNADIMR/L	3	639.43	-0.103	11.6	6.84E-28	169-184	2	.....
										R/MEENFALEAANYODITGR/L	3	734.36	-0.028	11.54	1.19E-27	346-364	0	.....
										R/MEENFALEAANYODITGR/L	2	1101.6	-0.586	11.48	1.54E-27	346-364	0	.....
										K/LOEEMLOREAEASTLOSFR/O	3	775.83	-0.452	11.48	2.15E-27	189-207	1	.....
										R/DNLADIMR/L	2	539.74	-0.984	11.41	7.35E-27	176-184	0	.....
										R/ROVDOLNDRAR/V	3	482.02	-0.093	11.31	2.55E-26	159-170	2	.....

































355	Alpha-endase	ENO1	P04764	LC-ESI-IT	172.2	34	34	18	48%	KVNOIGSVTESLOACK/L	2	815.52	1.894	12.96	1.08E-35	343-357	0	.....Oys CAM:.....
										R/AVPSGASTGTEALELR/D	2	903.02	-0.044	12.68	1.43E-34	32-49	0	.....
										KDYPMVSTEDPFDDWDWAKOF	2	1285.1	-0.506	12.59	4.13E-34	285-305	0	.....Oys CAM:.....
										KVNOIGSVTESLOACK/L	2	817.88	-0.466	12.58	5.44E-34	343-357	0	.....Oys CAM:.....
										R/GNPTVEVDLYTAK/G	2	703.8	0.062	12.44	3.69E-33	15-27	0	.....Oxidation M:.....
										K/AGYTDWIGNDVAASEFYR/A	2	1104	0.478	12.39	3.72E-33	233-252	0	.....
										K/DOLMIENDGTENK/S	2	818.85	0.031	11.88	3.41E-30	89-102	0	.....
										R/GNPTVEVDLYTAK/G	2	703.83	0.032	11.43	6.75E-28	15-27	0	.....
										K/DATNVGDEGGFAPNILENK/E	3	653.83	0.483	11.42	9.07E-28	202-220	0	.....Oxidation M:.....
										R/AGYTDWIGNDVAASEFYR/A	2	1105	-0.512	11.34	1.14E-27	233-252	0	.....
										K/DATNVGDEGGFAPNILENK/E	2	903.03	-0.054	11.17	9.68E-27	32-49	0	.....
										R/GNPTVEVDLYTAK/G	2	703.74	0.122	10.67	2.81E-24	202-220	0	.....
										K/DOLMIENDGTENK/S	2	818.36	0.521	10.46	2.71E-23	89-102	0	.....
										KVNOIGSVTESLOACK/L	2	817.43	-0.016	10.15	6.69E-22	343-357	0	.....Oys CAM:.....
										R/AGYTDWIGNDVAASEFYR/A	2	1096.6	-0.05	9.93	4.66E-21	233-252	0	.....
										K/DOLMIENDGTENK/S	2	826.87	0.009	9.73	4.61E-20	89-102	0	.....Oxidation M:.....
										R/YITPDOLADLYK/S	2	720.39	-0.016	9.71	6.00E-20	269-280	0	.....
										KVNOIGSVTESLOACK/L	2	816.6	0.814	9.23	1.52E-17	343-357	0	.....Oys CAM:.....
										K/DOLMIENDGTENK/S	2	834.84	0.036	9.16	1.09E-17	89-102	0	.....Oxidation M:.....
										R/JGAEYHNLK/N	2	572.29	0.021	8.94	8.86E-17	183-192	0	.....
										K/LNVFOEKDOLMIENDGTENK/S	3	870.32	-0.232	8.77	3.35E-16	81-102	1	.....Oxidation M:.....
										K/DOLMIENDGTENK/S	2	826.83	0.049	8.6	1.62E-15	89-102	0	.....Oxidation M:.....
										K/DOLMIENDGTENK/S	2	826.9	-0.021	8.35	1.49E-14	89-102	0	.....Oxidation M:.....
										K/SCNLLK/N	2	504.19	0.064	8	3.17E-13	335-342	0	.....Oys CAM:.....
										K/ETATAGIQWGDLLTYNPK/R	2	1024	0.039	7.78	9.59E-13	306-325	0	.....
										K/DATNVGDEGGFAPN/I	2	682.26	0.032	7.76	1.73E-12	202-215	half	.....
										KYNOLLRI	2	403.73	0	7.64	6.03E-12	406-411	0	.....
										R/AGKYDLDKSPDDASR/Y	3	595.59	0.03	7.08	4.30E-10	253-268	2	.....
										I/APALYSK/K	1	685.38	0.044	6.87	7.12E-10	73-79	half	.....
										I/GAEYHNLK/N	2	515.76	0.009	6.78	3.12E-09	184-192	half	.....
										R/AGYTDWIGNDVAASEFYR/A	3	731.64	-0.291	6.55	3.28E-08	233-252	0	.....
										V/NGMDVAASEFYR/A	2	736.91	0.446	6.46	1.85E-08	240-252	half	.....Oxidation M:.....
										R/GNPTVEVDLYTAK/G	1	1406.8	-0.064	6.31	3.42E-08	15-27	0	.....
355	Alpha-endase	ENO1	P04764	LC-ESI-Q-TOF	no													
381	Lamin-A	LUNA	P48679	LC-ESI-IT	446.7	90	90	48	50%	R/TALNATGEEVAMR/K	2	738.4	-0.02	14.09	1.17E-42	528-541	0	.....Oxidation M:.....
										R/SGAASSTPLSPTR/I	2	746.42	-0.043	13.72	1.68E-40	528-541	0	.....
										R/NSNLVGAHHEELOOSR/I	2	680.37	-0.073	12.49	1.98E-33	Dec-25	0	.....
										R/LOEKEDLOELNDR/I	2	877.01	-0.075	12.33	1.10E-32	281-296	0	.....
										R/MOODLOEYOEIIDLK/I	2	815.45	-0.042	12.25	3.94E-32	29-41	1	.....
										R/ITSEEVSR/E	2	955.57	-0.106	11.93	1.23E-30	352-366	0	.....Oxidation M:.....
										R/ITSEEVSR/E	2	574.79	0.003	11.9	8.74E-30	63-72	0	.....
										R/ITSEEVSR/E	2	574.82	-0.027	11.64	6.73E-29	63-72	0	.....
										R/MOODLOEYOEIIDLK/I	2	947.55	-0.083	11.59	7.82E-29	352-366	0	.....
										R/NSNLVGAHHEELOOSR/I	2	877.01	-0.075	11.57	1.01E-28	281-296	0	.....
										R/IRDELSLR/E	2	594.37	-0.07	11.03	6.09E-26	320-329	1	.....
										R/AVVEVEEGKFR/I	2	803.43	-0.02	10.99	1.02E-25	440-453	1	.....
										R/LOEKEDLOELNDR/I	2	814.94	0.468	10.99	8.43E-26	29-41	1	.....
										K/FAALSTALSEK/R	2	560.34	-0.042	10.75	1.70E-24	145-155	0	.....
										R/SLTENAGR/I	2	545.3	-0.02	10.61	7.13E-24	51-60	0	.....
										R/ISLSAQLSOLK/O	2	715.95	-0.054	10.44	3.56E-23	299-311	0	.....
										K/LEALGEAK/K	2	449.89	1.363	10.36	2.05E-22	172-180	0	.....
										R/SLTMVEDNDDEEDGDELLH/H	2	1229.6	-0.055	10.31	8.93E-23	546-566	half	.....Oxidation M:.....
										R/ITLEGELNDR/G	2	591.86	-0.051	10.2	4.29E-22	157-166	0	.....
										ANVEVEDEGKFR/I	2	718.39	-0.033	10.13	8.18E-22	442-453	half	.....
										K/KEGDLLAAOAR/I	2	586.31	0.015	10.07	1.67E-21	123-133	1	.....
										R/SLTENAGR/I	2	545.35	-0.07	10.07	1.88E-21	51-60	0	.....
										R/NSNLVGAHHEELOOSR/I	2	877.44	-0.505	10.06	1.30E-21	281-296	0	.....
										K/AAVEAELGDARK/I	2	647.28	0.045	9.96	1.45E-20	79-90	1	.....
										S/NLNVGAHHEELOOSR/I	2	715.75	0.647	9.9	8.74E-21	283-296	half	.....
										K/FAALSTALSEK/R	2	560.32	-0.022	9.76	4.58E-20	145-155	0	.....





381	Lamin-A	LMNA	P48679	LC-ESI-Q-TOF	59.3	7	7	7	12%	R/LOEKEDLOELNDR/L R/AOHEDOVOYK/K R/SVGGSGGSGFDMVLR/S	2 3 2	815.48 458.55 783.88	-0.072 0.331 0.002	6.07 6 10.63	3.00E-07 250-260 4.24E-24	29-41 629-645	1 0 0	..... ..... .....
										R/KOLEALNLSK/E R/TI EGI HDLRGQVAKI F/A R/TI LEGI HDLRGQVAKI/L R/SQAASSTPLSPTR/I R/IDSLSAOLSOLOK/O R/ADALOELR/A	2 3 3 2 2 2	622.37 636.67 555.97 680.84 716.38 514.8	-0.002 0.007 -0.003 -0.495 -0.479 -0.008	9.69 9.17 8.26 7.73 6.95 6.9	7.42E-20 1.19E-17 4.64E-14 2.42E-12 7.17E-10 1.39E-09	134-144 157-173 157-171 Dec-25 299-311 241-249	1 half 1 0 0 0	..... ..... ..... ..... ..... .....
382	Tropomyosin alpha-1 chain	TPM1	P04692	LC-ESI-IT	101.5	20	20	9	25%	K/ADSLSLAADETA/K/A R/TOLVEEELDR/A K/AEADVASLNR/R K/LVIESDLR/A R/VLEELHKAEDSLAADETA/K/A K/LVIESDLR/A R/TOLVEEELDR/A R/TOLVEEELDR/A R/AOKDEEKMEIOELK/E R/AOKDEEKMEIOELK/E R/AOKDEEKMEIOELK/E R/VLEELHKAEDSLAADETA/K/A K/MEIOELK/E R/KLVIESDLRAEER/A R/KLVIESDLRAEER/A K/MEIOELK/E R/KLVIESDLRAEER/A K/LVIESDLRAEER/A R/TOLVEEELDR/A	2 2 2 2 3 3 2 2 3 3 3 3 2 2 1 3	702.91 623.37 523.37 593.95 731.9 594.4 622.89 622.42 659.48 654.15 654.08 659.42 752.87 566.47 601.19 601.18 574.44 836.68 558.15 1243.9	-0.064 -1.04 -1.03 -0.112 -0.175 -0.562 -0.56 -0.09 -0.142 -0.143 -0.073 -0.082 -1.145 -0.162 -0.523 -0.513 -0.135 -0.231 -0.181 -0.207	16.23 12.97 12.6 12.28 12.2 12.03 11.25 11.11 10.6 10.51 10.41 9.52 9.2 8.8 8.42 8.16 7.8 7.41 6.73 6.2	7.61E-57 1.15E-35 5.98E-34 2.87E-32 7.02E-32 1.58E-30 5.45E-27 2.54E-26 2.04E-23 2.34E-23 6.56E-23 4.74E-19 8.73E-18 2.52E-14 2.27E-13 1.66E-12 2.38E-11 4.67E-09 6.66E-08	67-80 92-101 81-90 169-178 60-80 169-178 92-101 92-101 134-149 134-149 134-149 134-149 60-80 141-149 168-182 168-182 141-149 169-182 169-182 60-80	0 0 0 0 1 0 0 0 2 2 2 2 1 0 2 2 0 1 0 1	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
382	Tropomyosin alpha-1 chain	TPM1	P04692	LC-ESI-Q-TOF	81.2	8	8	8	36%	R/VLEELHKAEDSLAADETA/K/A K/CAELEELKTVTNNL/K/S R/SKOLEDISAK/E K/LEAKKADESER/G R/TOLVEEELDR/A R/AOKDEEKMEIOELK/E K/AEADVASLNR/I R/TOLVEEELDR/A R/IOELVEEELDR/A K/DGQVIGIGAGQGSN/I	3 3 2 3 3 3 3 3 2 2	751.72 630.99 624.33 492.91 576.64 659.34 401.22 628.67 693.35	0.009 -0.005 -0.004 -0.009 -0.002 -0.003 -0.002 -0.002 0.01	15.81 12.21 10.94 9.99 9.43 8.84 6.99 6.97	4.92E-54 7.18E-32 1.82E-25 5.26E-21 1.08E-18 2.35E-16 9.12E-10 7.30E-10 2.70E-42	60-80 190-205 36-46 113-125 92-105 134-149 81-91 91-105 438-451	1 1 1 1 1 2 1 2 0	..... ..... ..... ..... ..... ..... ..... ..... ..... .....
392	Bifunctional purine biosynthesis protein PI RH (ATTC)	ATTC	O35567	LC-ESI-IT	68.8	12	12	7	19%	R/GAVDIPAAAFK/H R/DVSELTGPEMLGR/V R/DVSELTGPEMLGR/V R/MSSFGDFVSLDVCVPTAK/I R/GAVDIPAAAFK/H K/NGSYCVLWMDOSYKPDENEVR/T R/MSSFGDFVSLDVCVPTAK/I R/EVSDGVAPGTEEEAK/I R/NIPEDAADMAR/L K/NGSYCVLWMDOSYKPDENEVR/T K/DGQVIGIGAGQGSN/I	2 2 2 2 3 2 2 2 3 2	574.37 804.41 812.42 1081.6 573.83 845.34 1082.1 903.51 609.75 843.97 693.91	-0.559 -0.02 -0.032 -0.087 -0.019 -0.631 -0.567 -0.066 0.025 0.739 -0.55	10.82 10.29 9.78 9.5 9.07 8.88 8.81 8.26 7 6.93 6.45	6.69E-25 1.42E-22 2.52E-20 3.16E-19 3.11E-17 1.10E-16 1.89E-16 2.85E-14 5.77E-10 2.37E-09 1.99E-08	255-266 50-64 50-64 312-331 255-266 359-379 312-331 336-352 80-90 359-379 438-451	0 0 0 0 0 0 0 0 0 0 0	..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
392	Bifunctional purine biosynthesis protein PI RH (ATTC)	ATTC	O35567	LC-ESI-Q-TOF	no													
397	Tropomyosin alpha-1 chain	TPM1	P04692	LC-ESI-IT	311.5	59	59	32	64%	K/KATDAEADVASLNR/R R/SKOLEDVSLQK/K R/LATALOKLEAAKAADESER/G K/LVIESDLER/A	2 2 3 2	731.2 759.31 735.03 594	-0.329 -0.396 -0.317 -0.162	15.75 14.93 14.36 13.75	1.48E-53 4.76E-48 2.17E-44 1.15E-40	77-90 36-48 106-125 169-178	1 1 2 0	..... ..... ..... .....





400	Phosphoglycerate mutase 1	PGAM1	P25113	LC-ESI-IT	91.8	18	18	9	42%	R/V/LA/HG/SLR/G	2	576.36	-0.522	12.45	3.29E-33	180-190	0	.....
										R/HGSAWNLNR/F	2	656.75	0.055	11.69	9.53E-29	Oct-20	0	.....
										R/DAGYEDICTSVOK/R	2	891.41	-1.012	11.02	6.39E-26	46-60	0	.....:Ovs CAM:.....
										R/DAGYEDICTSVOK/R	2	889.81	0.588	10.95	1.25E-25	46-60	0	.....:Ovs CAM:.....
										R/HGSAWNLNR/F	2	656.76	0.045	10.93	2.01E-25	Oct-20	0	.....
										R/FGSWYDADLSPAGHEAK/R	3	660.63	0.001	10.67	4.93E-24	21-38	0	.....
										R/YADLTEDOLPSCSLK/D	2	934.91	0.023	10.01	2.84E-21	141-156	0	.....:Ovs CAM:.....
										K/AMEAFAAOGK/V	2	488.25	0	9.69	9.52E-20	241-250	0	.....
										R/FGSWYDADLSPAGHEAK/R	2	990.45	-0.008	9.56	2.74E-19	21-38	0	.....
										R/VLAHAGNSLR/G	2	575.79	0.048	9.52	4.49E-19	180-190	0	.....
										M/OFLGDEETV/K	2	597.31	-0.017	9.29	4.15E-18	230-239	half	.....
										R/KAMEAFAAOGK/V	2	552.29	0.008	9.09	2.37E-17	240-250	1	.....
										R/FGSWYDADLSPAGHEAK/R	2	990.43	0.012	9.04	3.48E-17	21-38	0	.....
										R/YADLTEDOLPSCSLK/D	2	935.41	-0.477	8.26	8.38E-14	141-156	0	.....:Ovs CAM:.....
										R/ALPFWNEEVPQIK/E	2	842.45	0.009	7.88	1.67E-12	162-175	0	.....
										M/OFLGDEETV/K	2	597.26	0.033	7.63	6.30E-12	230-239	half	.....
										R/YADLTEDOLPSCSLK/D	3	623.64	-0.016	6.97	9.93E-10	141-156	0	.....:Ovs CAM:.....
										R/ALPFWNEEVPQIK/E	2	842.47	-0.011	6.1	2.13E-07	162-175	0	.....
400	Phosphoglycerate mutase 1	PGAM1	P25113	LC-ESI-Q-TOF	110.4	13	13	11	45%	R/T/LTWVLDADQMWLPVR/T	3	724.71	0.009	16.01	1.71E-55	65-82	0	.....:Oxidation M:.....
										R/FGSWYDADLSPAGHEAK/R	3	660.63	0.003	14.66	2.05E-46	21-38	0	.....
										R/T/LTWVLDADQMWLPVR/T	2	1086.5	0.039	14.32	1.95E-44	65-82	0	.....:Oxidation M:.....
										R/YADLTEDOLPSCSLKDTIAR/A	3	809.05	0.012	13.06	5.51E-37	141-161	1	.....:Ovs CAM:.....
										R/VLAHAGNSLR/G	2	575.83	0.011	10.5	2.36E-23	180-190	0	.....
										R/ALPFWNEEVPQIK/E	2	842.44	0.02	8.95	3.29E-17	162-175	0	.....
										R/FGSWYDADLSPAGHEAK/R/G	3	712.67	-0.001	8.68	7.33E-16	21-39	1	.....
										R/SYOVPPPPMPPHPPYSNISK/D	3	811.7	0.01	8.56	1.52E-15	117-137	0	.....
										R/HYGGLTGLNK/A	2	530.28	0.007	8.13	1.48E-13	90-99	0	.....:Oxidation M:.....
										L/IAHAGNSLR/G	2	469.76	0.006	7.88	8.21E-13	182-190	half	.....
										V/LAAGNSLR/G	2	526.3	0.008	7.05	4.80E-10	181-190	half	.....
										S/PAGHEAKR/G	2	497.76	0.001	6.88	1.05E-09	31-39	half	.....
										R/FGSWYDADLSPAGHEAKR/G	3	711.35	1.314	6.49	1.11E-08	21-39	1	.....
413	Ornithine aminotransferase	OAT	P04182	LC-ESI-IT	189.3	35	35	19	36%	K/GTYMWDEGR/Q	2	613.31	-0.023	14.63	3.62E-46	67-76	0	.....
										K/TEOGPPSSYEYER/E	2	820.42	-0.036	13.88	1.40E-41	33-46	0	.....
										K/KTEOGPPSSYEYER/E	2	884.51	-0.079	13.28	4.74E-38	32-46	1	.....
										L/APPLVIKEDIR/E	2	690.83	-0.432	12.54	8.44E-34	415-426	half	.....
										K/TEOGPPSSYEYER/E	2	820.89	-0.506	12.05	3.61E-31	33-46	0	.....
										K/GTYMWDEGR/Q	2	621.31	-0.025	11.69	2.97E-29	67-76	0	.....:Oxidation M:.....
										K/SDVDKLTLSR/A	2	624.34	0.011	11.62	7.71E-29	103-113	1	.....
										K/GTYMWDEGR/Q	2	621.28	0.005	11.53	1.92E-28	67-76	0	.....
										K/VLPMTGVEAGETACK/L	2	838.95	-0.047	11.41	6.80E-28	136-151	0	.....:Oxidation M:.....
										O/GPPSSYEYER/E	2	641.36	-0.051	11.33	1.50E-27	36-46	half	.....:Ovs CAM:.....
										R/IAALEVLEEHLAENADK/M	2	1089.6	-0.002	10.77	7.57E-25	332-351	0	.....
										R/APPLVIKEDIR/E	2	746.98	-0.04	10.72	1.65E-24	414-426	1	.....
										K/TEOGPPSSYEYER/E	2	820.42	-0.036	10.54	9.80E-24	33-46	0	.....
										K/LPSDWTAVR/G	2	528.82	-0.014	10.49	2.07E-23	363-372	0	.....
										R/IAALEVLEEHLAENADK/M	3	726.77	-0.056	10.11	1.14E-21	332-351	0	.....
										K/VLPMTGVEAGETACK/L	2	846.9	0	9.84	1.36E-20	136-151	0	.....
										K/KTEOGPPSSYEYER/E	2	884.97	-0.539	9.66	7.30E-20	32-46	1	.....:Oxidation M:.....:Ovs CAM:.....
										F/IADEIOTGLAR/T	2	593.8	0.025	9.27	3.70E-18	261-271	half	.....
										K/KTEOGPPSSYEYERESKY	3	705.01	-0.33	8.92	1.17E-16	32-49	2	.....
										A/PLVIKEDIR/E	2	654.9	-0.02	8.75	4.10E-16	416-426	half	.....
										A/PLVIKEDIR/E	2	654.89	-0.01	8.71	1.46E-15	416-426	half	.....
										L/APPLVIKEDIR/E	2	688.88	1.518	8.5	1.33E-15	416-426	half	.....
										F/IADEIOTGLAR/T	2	593.77	0.055	8.46	5.33E-15	261-271	half	.....
										G/STYGNPLGCR/I	2	591.23	0.042	7.85	9.01E-13	321-331	half	.....:Ovs CAM:.....
										K/SDVDKLTLSR/A	2	624.85	-0.499	7.79	1.44E-12	103-113	1	.....
										L/APPLVIKEDIR/E	2	688.91	1.488	7.63	4.06E-12	415-426	half	.....
										D/PGYIGVR/E	2	431.78	-0.037	7.48	1.99E-11	243-250	half	.....



413	Ornithine aminotransferase	OAT	P04182	LC-ESI-Q-TOF	151.2	19	13	35%	G/ETIPYNDLPALER/A	2	839.9	-0.472	7.34	3.92E-11	204-217	half	.....
									D/PGYLTGVR/E	2	430.14	1.603	7.02	1.27E-09	243-250	half	.....
									R/ESYEINKTK	2	466.23	0.028	6.57	1.34E-08	427-434	0	.....
									K/TEOGPPSSSEYIFER/E	3	590.3	-0.344	6.47	2.52E-08	32-46	1	.....
									R/ESYEINKTK	1	931.54	-0.031	6.46	1.43E-08	427-434	0	.....
									K/ELMKLPDSVAVR/G	3	525.37	-0.078	6.35	5.30E-08	359-372	1	.....
									V/ALERGGYGMWDVEGR/O	3	633.27	-0.617	6.08	7.71E-07	61-76	half	.....
									R/JAALAEVLEEHLAENADKMGAILR/K	4	709.62	0.011	15.65	7.63E-53	332-357	1	.....
									R/JAALAEVLEEHLAENADK/M	3	726.69	0.022	14.93	4.71E-48	332-351	0	.....
									K/GYMMWDVEGR/O	2	621.29	-0.001	13.48	5.08E-39	67-76	0	.....
421	Endoplasmatic reticulum protein 29	ERP29	P52555	LC-ESI-IT	94.8	17	11	38%	R/ATYNNVLGETEYITK/L	2	977.45	-0.486	12.62	2.69E-34	114-129	0	.....
									K/YGAHHPLPLVALER/G	3	579.63	0.003	12.41	7.26E-33	50-64	0	.....
									R/JAALAEVLEEHLAENADK/M	2	1089.6	0.008	12.28	1.61E-32	332-351	0	.....
									K/ELMKLPDSVAVR/G	3	525.29	0.003	11.84	7.30E-30	359-372	1	.....
									K/TEOGPPSSSEYIFER/E	2	820.85	-0.471	11.44	1.33E-28	33-46	0	.....
									K/PSDWTAVR/G	2	127.25	1.557	11.4	1.01E-27	363-372	0	.....
									K/PLPMNTGVEAGETACK/L	2	846.89	0.013	11.2	9.76E-27	136-151	0	.....
									A/PLVKEDEIR/E	3	436.92	0.002	11.16	2.12E-26	416-426	half	.....
									A/PLVKEDEIR/E	2	654.87	0.006	9.89	9.52E-21	416-426	half	.....
									R/DNGLAKPTHGDIR/L	3	540.64	-0.001	9.03	5.25E-17	399-413	0	.....
421	Endoplasmatic reticulum protein 29	ERP29	P52555	LC-ESI-IT	94.8	17	11	38%	R/NLAVDHNVRPOVLLGK/A	3	692.05	0.004	8.73	5.92E-16	275-292	0	.....
									K/PSDWTAVR/G	2	528.8	0.002	7.47	2.06E-11	363-372	0	.....
									K/ELMKLPDSVAVR/G	2	787.42	0.018	7.44	1.89E-11	359-372	1	.....
									L/PSDWTAVR/G	2	472.26	0	7.3	8.43E-11	364-372	half	.....
									K/PLPMNTGVEAGETACK/L	2	838.89	0.017	7.22	1.07E-10	136-151	0	.....
									R/NLAVDHNVRPOVLLGK/A	4	519.29	-0.003	7.11	3.89E-10	275-292	0	.....
									K/ILDQGEDFPASLAR/I	2	830.94	-0.028	12.11	8.02E-31	209-223	0	.....
									R/LAENSASSDLLVAEVSIDYGD/K/L	2	1234.7	-0.093	11.75	6.83E-29	76-99	0	.....
									K/ILDQGEDFPASLAR/I	2	830.94	-0.028	11.44	2.17E-27	209-223	0	.....
									K/GALPLDTVITK/V	2	662.88	-0.019	9.82	1.53E-19	37-48	0	.....
435	Troponin beta	TPM2	P58775	LC-ESI-IT	371.4	86	37	82%	K/FTDTPYYPGEK/O	2	624.27	0.013	9.63	1.08E-18	60-69	0	.....
									N/SASSDLLVAEVSIDYGD/K/L	3	1021	-0.026	9.04	2.20E-16	80-99	half	.....
									R/LAENSASSDLLVAEVSIDYGD/K/L	3	824.82	-1.426	8.98	1.07E-15	76-99	0	.....
									K/ILDQGEDFPASLAR/I	2	830.88	0.032	8.7	2.62E-15	209-223	0	.....
									R/DGDFENPVPYSAVK/V	2	797.89	-0.017	8.49	3.10E-14	123-137	0	.....
									K/FTDTPYYPGEKODEFR	2	947.99	-0.062	8.41	5.17E-14	60-74	1	.....
									R/LAENSASSDLLVAEVSIDYGD/K/L	3	823.75	-0.356	7.83	7.05E-12	76-99	0	.....
									K/ILDQGEDFPASLAR/I	2	831.39	-0.478	7.7	1.14E-11	209-223	0	.....
									R/DGDFENPVPYSAVK/V	2	797.91	-0.037	7.16	1.14E-09	123-137	0	.....
									R/DGDFENPVPY/S	2	576.39	0.356	6.61	2.18E-07	123-132	half	.....
421	Endoplasmatic reticulum protein 29	ERP29	P52555	LC-ESI-Q-TOF	50.8	6	6	24%	R/SLNLTAFR/K	2	519.01	-1.207	6.42	1.47E-07	243-251	0	.....
									K/FTDTPYYPGEKODEFR/L	3	684.36	-0.038	6.41	2.51E-07	60-75	2	.....
									K/WASOYLK/I	2	448.26	-0.023	6.08	1.17E-06	198-204	0	.....
									K/ILDQGEDFPASLAR/I	2	830.91	0.001	11.84	3.21E-30	177-191	0	.....
									R/DGDFENPVPYSAVK/V	2	797.87	0.002	9.12	1.01E-17	91-105	0	.....
									R/SLNLTAFR/K	2	581.86	-0.009	8.24	2.87E-14	211-220	1	.....
									D/ALAGOFIEASSR/E	2	625.33	0	7.44	1.62E-11	131-142	half	.....
									K/FTDTPYYPGEK/O	2	624.29	-0.007	7.35	3.49E-11	28-37	0	.....
									R/SLNLTAFR/K	2	517.81	-0.008	6.81	1.70E-09	211-219	0	.....
									K/ITDDI LEETLASAK/F	2	703.35	0.005	16.53	2.29E-59	252-264	0	.....
435	Troponin beta	TPM2	P58775	LC-ESI-IT	371.4	86	37	82%	K/KATDAADVASLN/R	2	731.37	-0.499	16.42	2.37E-58	77-90	1	.....
									K/SLAEEESTK/E	2	679.21	-0.38	16.38	5.89E-58	206-217	0	.....
									K/ITDDI LEETLASAK/E	2	703.4	-0.044	15.7	3.70E-53	252-264	0	.....
									R/LATALOKLEAEKADESER/G	3	734.74	-0.027	14.83	2.08E-47	106-125	2	.....
									R/LATALOKLEAEK/A	2	722.46	-0.054	13.55	1.38E-39	106-118	1	.....
									K/SLAEEESTK/E	2	678.94	-0.11	13.4	1.39E-38	206-217	0	.....
									R/KCOLLEEOOALOK/K	2	816.84	-0.433	13.14	3.83E-37	36-48	1	.....
									R/LATALOKLEAEKADESER/G	3	734.9	-0.187	12.68	1.63E-34	106-125	2	.....
									K/YSKXDAOFKLEAEK/K	3	661.69	-0.361	12.52	1.67E-33	60-76	2	.....
									K/YSKXDAOFKLEAEK/K	3	661.69	-0.361	12.52	1.67E-33	60-76	2	.....













539	Beta-actin	ACTB	P60711	LC-ESI-IT	24.5	5	5	2	10%	K/LCYVALDFEOMATAASSSSLEK/S	2	1283.7	-0.092	13.16	1.70E-37	215-237	0	.....Oxidation M:.....
										K/SYELPDGVITIGNER/F	2	896.03	-0.08	11.31	2.14E-27	238-253	0	.....
										K/LCYVALDFEOMATAASSSSLEK/S	3	856.21	-0.149	10.55	2.34E-23	215-237	0	.....Oxidation M:.....
										K/LCYVALDFEOMATAASSSSLEK/S	2	1277.2	-1.609	7.83	1.70E-12	215-237	0	.....Oys CAM:.....
										K/LCYVALDFEOMATAASSSSLEK/S	3	856.7	-0.639	7.27	1.76E-10	215-237	0	.....Oys CAM:.....
539	Beta-actin	ACTB	P60711	LC-ESI-Q-TOF	105.1	12	12	10	23%	R/LAMLQHEWSLGPISDLASTVR/S	3	794.74	0.011	19.1	1.85E-78	415-436	0	.....Oxidation M:.....
										KVQSLOAAVGTFSILR/S	2	859.97	0.005	13.24	3.89E-37	128-143	0	.....
										KVGAHGSEAVFR/D	3	453.25	-0.013	12.69	1.05E-33	349-361	0	.....
										K/SINDNIAFTDVOK/R	2	789.4	0.009	11.82	2.86E-29	213-226	0	.....
										KVQSLOAAVGTFSILR/S	3	573.65	0.007	11.79	5.14E-29	128-143	0	.....
										K/LSDGHWK/D	2	541.8	-0.005	10.01	1.32E-20	180-189	0	.....
										R/SLEETOLALSOLK/G	2	767.39	0.012	9.61	6.55E-19	437-450	0	.....
										K/IETNLSLESAK/G	2	667.84	-0.013	8.17	3.26E-13	527-538	0	.....
										K/GLLDDRLNDR/L	3	472.25	-0.002	7.02	3.17E-09	539-550	1	.....
										R/LSLDNLK/S	2	445.26	-0.005	6.8	1.49E-08	493-500	0	.....
										R/SEVLOK/L	2	408.75	-0.001	6.68	3.65E-08	166-172	0	.....
										K/GLLDDRLNDR/L	2	707.87	-0.001	6.15	7.05E-07	539-550	1	.....
542	Lamin-A	LMNA	P48679	LC-ESI-IT	269.5	40	40	75	42%	R/TALINATGEEVAMR/K	2	738.45	-0.07	14.98	2.33E-48	528-541	0	.....
										R/TALINATGEEVAMR/K	2	737.97	0.41	14.92	5.22E-48	528-541	0	.....
										R/TALINATGEEVAMR/K	2	738.47	-0.09	14.32	3.88E-44	528-541	0	.....
										R/GSHGSSGPAETNLR/S	2	868.97	-0.102	14.13	4.98E-43	569-584	0	.....Oys CAM:.....
										R/ITESEWSR/E	2	574.9	-0.107	14.11	7.84E-43	63-72	0	.....
										R/SVGGSGGSGFQNLVTR/S	2	783.96	-0.081	13.81	3.76E-41	629-645	0	.....
										R/TALINATGEEVAMR/K	2	746.42	-0.043	13.67	3.57E-40	528-541	0	.....Oxidation M:.....
										R/NSLVGAHHELOOSR/I	2	877.01	-0.075	12.93	5.08E-36	281-296	0	.....
										R/ITESEWSR/E	2	574.93	-0.137	12.68	1.76E-34	63-72	0	.....
										R/SGAASSTLSPTR/I	2	680.55	-0.203	12.64	2.74E-34	Dec-25	0	.....
										R/VAEVEDEEGK/F	2	602.43	-0.139	12.1	2.38E-31	440-450	0	.....
										R/TVLCTGCGPADK/A	2	703.92	-0.096	11.86	3.87E-30	587-599	0	.....Oys CAM:.....
										K/EAMSLTALSEK/R	2	560.44	-0.142	11.78	1.10E-29	145-155	0	.....
										R/SLETNAGLR/L	2	545.39	-0.11	11.41	8.42E-28	51-60	0	.....
										R/LOEKEDLOELNDR/L	2	815.48	-0.072	11.33	1.85E-27	29-41	1	.....
										R/DSLSAOLSOLOK/O	2	716	-0.104	11.31	2.29E-27	299-311	0	.....
										R/SLETNAGLR/L	2	545.4	-0.12	10.98	1.04E-25	51-60	0	.....
										R/MOOLDEYOELLDIK/L	2	953.58	-0.116	10.97	9.45E-26	352-366	0	.....Oxidation M:.....
										K/AYEALGDAR/K	2	583.4	-0.122	9.97	4.22E-21	79-89	0	.....
										R/TLELGHDLR/G	2	592.06	-0.251	9.81	2.21E-20	157-166	0	.....
										K/DLEALLNSK/E	2	501.91	-0.133	9.53	3.90E-19	136-144	0	.....
										R/TLELGHDLR/G	2	592.03	-0.221	9.49	5.00E-19	157-166	0	.....
										R/LSPTSOR/S	2	486.81	-0.051	9.2	9.80E-18	389-397	0	.....
										R/LAVYTDNR/V	2	425.39	-0.145	9.17	1.26E-17	42-48	0	.....
										K/NTYSEELR/E	2	512.39	-0.131	8.99	6.21E-17	209-216	0	.....
										R/LVEIDNGK/O	2	444.34	-0.095	8.96	8.58E-17	226-233	0	.....
										R/LAVYTDNR/V	2	425.35	-0.105	8.88	1.76E-16	42-48	0	.....
										K/EGDLAAQAR/L	2	522.4	-0.122	8.85	2.08E-16	124-133	0	.....
										R/LADALOELR/A	2	514.87	-0.08	8.77	4.93E-16	241-249	0	.....
										R/LADALOELR/A	2	514.99	-0.2	8.55	3.45E-15	241-249	0	.....
										R/SGAASSTLSPTR/I	2	680.94	-0.593	8.28	2.64E-14	Dec-25	0	.....
										K/LEALGEAK/K	2	451.41	-0.157	8.12	1.31E-13	172-180	0	.....
										R/LOLELSK/V	2	415.85	-0.097	7.59	1.02E-11	102-108	0	.....
										R/LOEKEDLOELNDR/L	3	544.07	-0.129	7.35	5.68E-11	29-41	1	.....
										R/LOLELSK/V	2	415.91	-0.157	7.16	2.45E-10	102-108	0	.....
										R/EMEMARMOOOLDEYOELLDIK/L	3	956.27	0.524	7.13	1.64E-10	344-366	2	.....
										R/LVEIDNGK/O	2	444.82	-0.575	6.38	4.90E-08	226-233	0	.....
										R/TLELGHDLR/G	3	394.92	-0.045	6.3	9.33E-08	157-166	0	.....
										R/LADALOELR/A	1	1028.7	-0.097	6.26	6.24E-08	241-249	0	.....
542	Lamin-A	LMNA	P48679	LC-ESI-Q-TOF	no					K/AYEALGDAR/K	1	1165.7	-0.112	6.16	9.00E-08	79-89	0	.....
543	Adenylyl cyclase-associated protein_1	CAP1	Q08163	LC-ESI-IT	no													



543	AdenylM cyclase-associated protein 1	CAP1	Q08163	LC-ESI-Q-TOF	132.7	14	14	39%	K/FNFHLSVSEISQALGWVLAAK/P	3	820.43	0.015	13.67	2.86E-40	125-147	half	.....
									K/LSDLLAPISQIOEVITR/E	2	1087.1	-0.475	12.26	2.11E-32	99-117	0	.....
									R/AYLSWTLOAYIK/E	2	849.95	0.008	11.69	2.44E-29	183-196	0	.....
									R/VNCEVSNLVDDTELK/Q	2	1030	0.018	11.38	7.51E-28	329-346	0	.....
									K/NSLDCEVSAK/S	2	618.3	0.001	10.58	9.14E-24	421-431	0	.....Oys CAM.....
									R/SALFAQINOGESITHALK/H	3	643.34	0.008	10.47	2.78E-23	253-270	0	.....
									K/LGLVDDWVGIVINSR/D	2	979.52	0.035	9.61	1.36E-19	376-393	0	.....
									K/TDGGCHAYLSK/N	2	576.27	-0.012	9.28	4.12E-18	411-420	0	.....Oys CAM.....
									R/ALLVTASOCOPAGNKL	2	843.93	-0.489	9.07	1.92E-17	83-98	0	.....Oys CAM.....
									K/EFHTGLAWSKT	2	638.82	0.001	8.24	3.29E-14	197-207	0	.....
									S/NLVDDTELK/Q	2	580.31	0.005	7.29	8.79E-11	337-346	half	.....
									F/SAPKQTSPPSPATK/K	3	541.63	-0.331	6.97	9.23E-10	299-314	half	.....
									K/KEPALLEGK/K	2	613.85	0.003	6.15	1.49E-07	315-325	1	.....
									K/EPALLEGK/K	2	549.81	0	6.09	2.04E-07	316-325	0	.....
552	Heat shock protein beta-1	HSPB1	P42930	LC-ESI-IT	51.4	13	6	23%	R/QLSGGVSEIR/Q	2	538.21	0.081	10.66	4.10E-24	84-93	0	.....
									R/QLSGGVSEIR/Q	2	538.03	0.261	9.63	1.46E-19	84-93	0	.....
									R/DWYPAHSRL	2	516.17	0.068	9.5	5.54E-19	21-28	0	.....
									R/DWYPAHSRL	2	516.29	-0.052	8.63	1.52E-15	21-28	0	.....
									R/LFDOAFGVP/R	2	575.3	0.006	8.22	4.59E-14	29-38	0	.....
									K/TKEGVETGK/H	2	580.8	0.03	8.17	7.57E-14	117-127	1	.....
									R/QLSGGVSEIR/Q	2	578.25	0.024	7.77	1.97E-12	84-93	0	.....PHOS.....
									K/EGVETGK/H	2	466.17	0.088	7.62	6.43E-12	119-127	0	.....
									R/LFDOAFGVP/R	2	575.3	0.006	7.53	3.74E-11	29-38	0	.....
									R/SPSWEP/R	2	503.23	0.013	7.24	1.19E-10	13-20	0	.....
									R/QLSGGVSEIR/Q	2	577.75	0.524	7.19	1.43E-10	84-93	0	.....PHOS.....
									R/RVPP/S	1	518.28	0.029	6.44	9.87E-08	05-Aug	half	.....
									R/DWYPAHSRL	2	516.29	-0.052	6.04	1.21E-06	21-28	0	.....
552	Heat shock protein beta-1	HSPB1	P42930	LC-ESI-Q-TOF	68.2	7	7	33%	R/VSLDVNHFAPEELTVK/T	3	599.99	-0.006	12.9	1.30E-35	101-116	0	.....
									R/AQIGGPESEOSGAK/-	2	679.83	-0.003	11.7	2.91E-29	193-206	0	.....
									K/AVTQAEITPVTEAR/A	2	916.98	0.013	11.06	3.42E-26	176-192	0	.....
									R/VSLDVNHFAPEELTVK/T	2	850.44	-0.499	8.93	7.12E-17	102-116	half	.....
									R/LFDOAFGVP/R	2	575.31	-0.008	8.43	7.97E-15	29-38	0	.....
									S/LVDVNHFAPEELTVK/T	2	806.41	0.012	8.01	2.39E-13	103-116	half	.....
									E/GTLTVAPLPA/A	2	563.32	0.013	7.13	2.01E-10	165-175	half	.....
562	Proteasome subunit beta type 4	PSMB4	P34067	LC-ESI-IT	58.4	15	6	26%	-/TQNPMTGTSLVGK/F	2	774.43	-0.021	12.27	2.70E-32	Jan-15	0	.....Oxidation M.....
									R/VNDSTMLGASGDYADFOLK/Q	2	1106.1	-0.131	12.22	3.52E-32	45-64	0	.....Oxidation M.....
									-/TONPMTGTSLVGK/F	2	766.46	-0.049	11.07	4.07E-26	Jan-15	0	.....
									K/QPVLQSOTEAR/E	2	564.74	0.064	11.02	7.69E-26	157-166	0	.....
									K/QPVLQSOTEAR/E	2	564.81	-0.006	10.92	2.20E-25	157-166	0	.....
									R/VNDSTMLGASGDYADFOLK/Q	2	1098.2	-0.148	10.61	1.22E-23	45-64	0	.....
									K/VIGGY/A	1	508.25	0.027	8.77	1.13E-16	111-115	half	.....
									-/TONPMTGTSLVGK/F	2	766.94	-0.529	8.17	6.80E-14	Jan-15	0	.....
									R/FOVATVTEK/G	2	511.76	0.019	7.91	7.75E-13	187-195	0	.....
									R/EVLEKQPVLSOTEAR/E	3	576.01	0.306	7.79	1.75E-12	152-166	1	.....
									-/TONPMTGTSLVGK/F	2	766.43	-0.019	7.23	1.13E-10	Jan-15	0	.....
									R/EVLEKQPVLSOTEAR/E	3	576.72	-0.404	7.2	4.24E-10	152-166	1	.....
									R/AHSHWLTR/A	2	492.28	-0.005	7.17	1.62E-10	87-94	0	.....
									-/TONPMTGTSLVGK/F	2	774.51	-0.101	7.14	1.94E-10	Jan-15	0	.....
									K/QPVLQSOTEAR/E	2	565.24	-0.436	6.39	3.85E-08	157-166	0	.....Oxidation M.....
562	Proteasome subunit beta type 4	PSMB4	P34067	LC-ESI-Q-TOF	53.6	5	5	22%	K/QPVLQSOTEAR/E	2	564.8	0.009	12.96	1.03E-35	157-166	0	.....
									R/EVLEKQPVLSOTEAR/E	3	576.31	0.004	11.7	4.27E-29	152-166	1	.....
									R/VNDSTMLGASGDYADFOLK/Q	2	1106	0.013	10.63	4.70E-24	45-64	0	.....Oxidation M.....
									-/TONPMTGTSLVGK/F	2	774.4	0.014	10.27	2.90E-22	Jan-15	0	.....Oxidation M.....
									R/AHSHWLTR/A	2	492.27	0.003	8.02	3.16E-13	87-94	0	.....
565	Tropomyosin alpha-4	TPM4	P09495	LC-ESI-IT	287.2	82	29	73%	R/KIQALQQQADDAEDR/A	2	865	-0.071	14.08	1.34E-42	Dec-26	1	.....
									R/RIOLVFEELDR/A	2	700.45	-0.069	13.96	7.16E-42	54-64	1	.....





565	Tropomyosin alpha-4	TPM4	P09495	LC-ESI-Q-TOF	98.6	12	10	57%	R/KLVILEGELAEER/A	3	595.76	-0.425	6.92	1.60E-09	131-145	2	.....	.....
									R/KLVILEGELAEER/A	3	595.32	0.015	6.86	2.47E-09	131-145	2	.....	.....
									K/SSDLEELKNVTNNL/K/S	2	918.6	-1.635	6.7	4.57E-09	153-168	1	.....	.....
									K/EEVNGVHOTDNLN/C	2	1034.6	-0.567	6.68	1.39E-08	228-245	half	.....	.....
									R/KYEEVAR/K	2	447.84	-0.102	6.66	7.49E-09	124-130	1	.....	.....
									K/YSEKDYKEIK/L	3	563.72	0.213	6.64	1.15E-08	177-189	2	.....	.....
									K/SSDLEELKNVTNNL/K/S	2	917.54	-0.575	6.57	1.17E-08	153-168	1	.....	.....
									R/AMKDEKMEIOEMOLK/E	4	500.24	-0.25	6.55	2.53E-08	97-112	2	.....	Oxidation M:.....
									K/HIAEADRYEEVAR/K	4	454.85	-0.12	6.49	3.93E-08	116-130	2	.....	.....
									K/YSEKDYKEIK/L	3	564.08	-0.147	6.47	3.47E-08	177-189	2	.....	.....
565	Tropomyosin alpha-4	TPM4	P09495	LC-ESI-Q-TOF	98.6	12	10	57%	K/VILEGELAEER/A	2	828.53	-0.078	6.37	5.01E-08	132-145	1	.....	.....
									K/EEVNGVHOTDNLN/L	2	920.62	-0.18	6.34	4.79E-08	228-245	half	.....	.....
									R/KVILEGELAEER/A	3	595.63	-0.295	6.31	1.01E-07	131-145	2	.....	.....
									R/KVILEGELAEER/A	3	551.37	1.267	6.28	1.31E-07	132-145	1	.....	.....
									R/TOLVEELDRA/A	1	1243.8	-0.097	6.27	1.58E-07	55-64	0	.....	.....
									-IAGNLSAVR/K	2	600.94	-0.599	6.09	3.35E-07	01-Nov	1	ACET interm:.....	.....
									-IAGNLSAVR/K	2	601.12	-0.779	6.07	3.76E-07	01-Nov	1	ACET interm:.....	.....
									E/IAKADESER/G	2	551.57	1.69	6.06	4.07E-07	79-88	half	.....	.....
									K/SSDLEELKNVTNNL/K/S	3	611.64	0.009	14.49	3.53E-45	153-168	1	.....	.....
									K/SSDLEELKNVTNNL/K/S	2	916.95	0.018	11.77	9.42E-30	153-168	1	.....	.....
572	Elongation factor 1-gamma	EF1G	Q68FR6	LC-ESI-IT	59.2	10	6	15%	K/LAOKAENGLHOTDNLNLCI/-	3	951.14	0.011	11.3	2.35E-27	223-247	1	.....	.....
									K/TOLAOOADAEDRAAGLOR/E	3	752.04	0.007	11.07	3.67E-26	13-32	1	.....	.....
									K/HIAEADRYEEVAR/K	4	454.73	-0.002	10.82	9.72E-25	116-130	2	.....	.....
									R/KVILEGELAEER/A	3	595.33	0.008	9.66	1.28E-19	131-145	2	.....	.....
									R/TOLVEELDRAOER/L	3	576.62	0.014	9.6	2.18E-19	55-68	1	.....	.....
									K/LEAKAADSERV/G	3	492.9	-0.004	8.39	1.50E-14	76-88	1	.....	.....
									-IAGNLSAVR/K	2	600.34	-0.001	8.34	2.04E-14	01-Nov	1	ACET interm:.....	.....
									R/EKAAGDAALNR/R	2	622.82	-0.006	7.76	2.09E-12	42-53	1	.....	.....
									E/IAKADESER/G	2	617.79	-0.004	7.16	1.93E-10	78-88	half	.....	.....
									R/EKAAGDAALNR/R	3	415.56	-0.01	6.45	3.96E-08	42-53	1	.....	.....
572	Elongation factor 1-gamma	EF1G	Q68FR6	LC-ESI-IT	59.2	10	6	15%	K/AAAPAFEEHDECEQALAAEPK/A	2	1179.1	-0.073	12.77	4.42E-35	253-274	0	.....	.....
									R/KDPGSEETTLVR/E	3	524.96	-0.014	10.47	3.32E-23	400-413	1	.....	.....
									-IAGTLTYPENWR/A	2	792.4	-0.022	10.25	2.40E-22	Jan-13	0	ACET interm:.....	.....
									K/AAAPAFEEHDECEQALAAEPK/A	2	1187.1	-0.055	10.06	1.52E-21	253-274	0	.....	Oxidation M:.....
									K/STFVLDFKR/K	2	621.37	-0.04	9.18	9.42E-18	285-294	1	.....	.....
									K/LDPGSEETTLVR/E	2	722.85	0.018	8.72	1.37E-15	401-413	0	.....	.....
									R/OAFPTNR/W	2	474.22	0.018	7.79	1.98E-12	181-188	0	.....	.....
									K/AAAPAFEEHDECEQALAAEPK/A	2	1188	-1.025	6.44	1.95E-08	253-274	0	.....	Oxidation M:.....
									R/OAFPTNR/W	2	474.23	0.008	6.44	3.34E-08	181-188	0	.....	.....
									K/AAAPAFEEHDECEQALAAEPK/A	2	1187.1	-0.035	6.25	7.60E-08	253-274	0	.....	Oxidation M:.....
572	Elongation factor 1-gamma	EF1G	Q68FR6	LC-ESI-Q-TOF	81.3	9	8	20%	K/STFVLDFKR/K	2	621.33	0.002	17.06	9.33E-63	285-294	1	.....	.....
									R/EYFSGEAFQHVGR/K/A	2	843.38	-0.49	11.21	7.76E-27	414-427	0	.....	.....
									R/VTLADITVWCTLLWY/K/O	2	1004.6	-0.005	10.59	6.76E-24	156-172	0	.....	.....
									R/KDPGSEETTLVR/E	3	524.95	-0.006	10.34	1.72E-22	400-413	1	.....	.....
									K/ALAAOYSGAQR/V	2	681.38	0.004	10.18	6.44E-22	17-29	0	.....	.....
									K/LDPGSEETTLVR/E	2	722.87	-0.004	8.33	2.20E-14	401-413	0	.....	.....
									N/ALAYYSNEELR/G	2	714.36	0.007	7.02	5.64E-10	73-84	half	.....	.....
									K/OVLEPSER/O	2	488.27	-0.007	6.54	2.16E-08	173-180	0	.....	.....
									R/KDPGSEETTLVR/E	2	786.9	0.014	6.25	1.02E-07	400-413	1	.....	.....
									K/QSFTMVADTPENLR/L	2	812.97	-0.085	13.85	2.56E-41	60-73	0	.....	Oxidation_M:.....
578	LIM and SH3 domain protein 1	LASP1	Q99MZ8	LC-ESI-IT	44.9	6	4	19%	K/OSFTMVADTPENLR/L	2	804.92	-0.032	12.02	5.51E-31	60-73	0	.....	.....
									K/GFSVADTPELOR/I	2	710.05	-0.183	11.94	1.51E-30	97-109	0	.....	.....
									K/TODDISNIK/Y	2	523.85	-0.073	10.26	2.77E-22	113-121	0	.....	.....
									R/TGDTGMLPANYEAL/-	1	776.49	-0.118	8.81	2.54E-16	249-263	0	.....	.....
									R/TGDTGMLPANYEAL/-	1	1567.8	-0.049	6.62	3.54E-09	249-263	0	.....	Oxidation_M:.....
									K/QSFTMVADTPENLR/L	2	812.88	0.002	10.05	1.42E-21	60-73	0	.....	Oxidation_M:.....
									K/QSFTMVADTPENLR/L	2	709.86	0.004	8.75	3.35E-16	97-109	0	.....	.....
									K/GFSVADTPELOR/I	2	709.86	0.004	8.75	3.35E-16	97-109	0	.....	.....
									K/GFSVADTPELOR/I	2	709.86	0.004	8.75	3.35E-16	97-109	0	.....	.....
									K/GFSVADTPELOR/I	2	709.86	0.004	8.75	3.35E-16	97-109	0	.....	.....



585	GTP-binding nuclear protein Ran	P62828	LC-ESI-IT	57.5	11	6	33%	K/FNWWDTAGQK/F	2	647.83	-0.023	11.99	9,10E-31	60-70	0	.....	.....
								K/LVVGDDGTGK/T	2	508.29	0.003	10.14	9,72E-22	Dec-22	0	.....	.....
								-/AAGEPQVDFK/L	2	622.86	-0.043	10.03	3,12E-21	01-Nov	0	.....	.....
								K/NLOYDYSK/S	2	607.78	0.026	8.85	2,04E-16	142-151	0	.....	.....
								-/AAGEPQVDFK/L	2	622.83	-0.013	8.85	2,22E-16	01-Nov	0	.....	.....
								-/AAGEPQVDFK/L	2	623.27	-0.453	8.63	1,81E-15	01-Nov	0	.....	.....
								K/LIGDNPILFVAMP/L	2	751.91	-0.028	8.28	2,54E-14	167-180	half	.....	.....
								R/VCEPIPLVCGNKV	2	758.35	0.037	8.25	3,27E-14	110-122	0	.....	.....
								R/VCEPIPLVCGNKV	2	758.89	-0.503	7.83	1,02E-12	110-122	0	.....	.....
								K/LIGDNPILFVAMP/L	2	751.93	-0.048	7.34	4,57E-11	167-180	half	.....	.....
								K/FNWWDTAGQK/F	2	648.37	-0.563	6.08	6,96E-07	60-70	0	.....	.....
585	GTP-binding nuclear protein Ran	P62828	LC-ESI-Q-TOF	47.8	5	5	31%	K/SYNFEPFLWLR/K	3	595.64	0	11.08	4,35E-26	152-165	0	.....	.....
								K/YVATLGVHPLVHFNK/G	3	684.7	0.002	11.06	4,09E-26	38-55	0	.....	.....
								R/VCEPIPLVCGNKV	2	758.39	-0.003	10.13	9,55E-22	110-122	0	.....	.....
								K/LVVGDDGTGK/T	2	508.29	0	8.12	1,58E-13	Dec-22	0	.....	.....
592	Vimentin	P31000	LC-ESI-IT	659.3	181	181	72%	K/NLOYDYSK/S	2	607.81	-0.007	7.39	4,62E-11	142-151	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1064.2	-0.654	16.68	8,60E-60	78-96	0	.....	.....
								K/FADLSEANR/N	2	547.41	-0.143	16.42	5,24E-58	294-303	0	.....	.....
								R/OVOSLTCEVDALK/G	2	746.29	-0.41	15.95	6,35E-55	321-333	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1063.7	-0.114	15.68	3,10E-53	78-96	0	.....	.....
								R/LGDLYEEMR/E	2	628.12	-0.333	15.46	2,85E-51	145-154	0	.....	.....
								R/LGDLYEEMR/E	2	635.87	-0.085	15.39	6,83E-51	145-154	0	.....	.....
								R/OVOSLTCEVDALK/G	2	745.97	-0.09	15.11	3,50E-49	321-333	0	.....	.....
								R/LGDLYEEMR/E	2	627.83	-0.043	14.87	2,26E-47	145-154	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1063.7	-0.124	14.86	9,86E-48	78-96	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1063.7	-0.134	14.43	5,11E-45	78-96	0	.....	.....
								R/LLODSVDFSLADINTEK/N	3	709.62	-0.26	14.43	7,67E-45	78-96	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1064.3	-0.224	14.42	2,01E-44	78-96	0	.....	.....
								R/DNLADIMR/L	2	539.74	-0.984	14.12	9,26E-43	175-183	0	.....	.....
								R/LLODSVDFSLADINTEK/N	3	709.35	0.01	14.1	8,96E-43	78-96	0	.....	.....
								R/LLODSVDFSLADINTEK/N	2	1063.8	-0.304	13.98	3,09E-42	78-96	0	.....	.....
								K/VESLOEIAFK/K	2	704.39	-1.008	13.91	1,30E-41	223-234	0	.....	.....
								R/EMENFALAAAYODITGR/L	2	1101.6	-0.636	13.64	1,11E-39	345-363	0	.....	.....
								R/OVOSLTCEVDALKGTNESLRO	3	845.22	-0.126	13.47	5,41E-39	320-341	2	.....	.....
								K/SKADLSEANR/N	2	654.92	-0.089	13.13	5,78E-37	292-303	1	.....	.....
								R/SRLGDLYEEMR/E	2	749.45	-0.096	13.1	8,00E-37	143-154	1	.....	.....
								R/KVESLOEIAFK/L	2	831.65	-0.173	12.98	4,18E-36	222-235	2	.....	.....
								K/FADLSEANR/N	2	546.87	0.397	12.81	5,48E-35	294-303	0	.....	.....
								R/EMENFALAAAYODITGR/L	2	1101	-0.036	12.79	3,07E-35	345-363	0	.....	.....
								R/KVESLOEIAFK/K	2	768.72	-1.29	12.7	3,51E-34	222-234	1	.....	.....
								R/ETNLESPLVDTH/S	2	734.43	-0.06	12.58	5,31E-34	424-436	half	.....	.....
								R/EMENFALAAAYODITGR/L	2	1109.1	-0.138	12.53	8,46E-34	345-363	0	.....	.....
								R/ODVDNASLR/L	2	544.82	-0.05	12.4	8,23E-33	207-216	0	.....	.....
								R/ODVDNASLR/L	2	544.81	-0.04	12.4	7,47E-33	207-216	0	.....	.....
								K/SRLGDLYEEMR/E	2	749.46	-0.106	12.31	1,98E-32	143-154	1	.....	.....
								R/OMRENFALAAAYODITGR/L	3	883.41	-0.015	12.22	8,18E-32	342-363	1	.....	.....
								K/MALDIEIATYR/K	2	656.86	-0.526	12.18	2,44E-31	390-400	0	.....	.....
								K/SRLGDLYEEMR/E	2	749.47	-0.116	12.07	3,98E-31	143-154	1	.....	.....
								K/NLOAEWYK/S	2	655.61	-0.303	11.91	2,59E-30	282-291	0	.....	.....
								R/ROVDLTNDKAR/V	3	482.02	-0.093	11.89	5,46E-30	158-169	2	.....	.....
								R/ETNLESPLVDTHS/K/R	2	842.39	-0.457	11.89	2,80E-30	424-438	0	.....	.....
								R/ROVDLTNDKAR/V	3	482.02	-0.093	11.8	1,55E-29	158-169	2	.....	.....
								R/ROVDLTNDKAR/V	3	482.04	-0.113	11.74	3,36E-29	158-169	2	.....	.....
								R/OMRENFALAAAYODITGR/L	3	878.11	-0.046	11.62	7,05E-29	342-363	1	.....	.....
								R/ETNKEVLOELNDR/F	2	794.45	-0.048	11.61	9,01E-29	100-112	1	.....	.....
								R/ETNKEVLOELNDR/F	2	794.48	-0.078	11.61	8,52E-29	100-112	1	.....	.....
								K/NLOAEWYK/S	2	655.35	-0.043	11.59	1,10E-28	282-291	0	.....	.....
								K/NLOAEWYK/S/F	2	762.96	-0.09	11.57	1,28E-28	282-293	1	.....	.....
								K/MALDIEIATYR/K	2	656.39	-0.056	11.56	1,53E-28	390-400	0	.....	.....
								R/ETNKEVLOELNDR/F	2	794.99	-0.588	11.55	1,73E-28	100-112	1	.....	.....







592	Vimentin	VIM	P31000	LC-ESI-Q-TOF	159.6	18	15	36%		R/ETNLESPLVDTHSKR/T	3	614.01	-0.352	6.43	9.00E-08	424-439	1	.....
										R/DNLAEDIMR/L	1	1076.6	-0.106	6.4	2.71E-08	175-183	0	.....
										K/FADLSEANRNDAIR/O	2	889.02	-0.085	6.35	4.48E-08	294-309	1	.....
										R/VOSSLTCEVDALGTNESLR/O	2	1189.7	-0.603	6.32	1.17E-07	321-341	1	.....:Oys CAM:.....
										D/NLAEDIMR/L	2	479.94	1.302	6.28	1.13E-07	176-183	half	.....
										R/EKLOEMLOREAEESTLOSFR/O	4	646.34	-0.27	6.25	1.24E-07	186-206	2	.....
										R/LGDIYEEMRELR/R	2	834.95	-0.051	6.15	1.59E-07	145-157	1	.....:Oxidation M:.....
										K/SKFDLSEANRNDAIR/O	3	664.77	-0.102	6.12	2.81E-07	292-309	2	.....
										R/LGDIYEEMRELR/R	3	557	-0.065	6.09	4.04E-07	145-157	1	.....:Oxidation M:.....
										R/ETNLESPLVDTH/S	2	734.33	0.04	6.09	2.33E-07	424-436	half	.....
										R/FANYIDKV	1	870.51	-0.074	6.07	1.42E-07	113-119	0	.....
										R/ETNLESPLVDTHSK/R	3	561.66	-0.035	6.03	5.26E-07	424-438	0	.....
K/RTLLIK/T	2	372.37	-0.109	6.02	6.06E-07	439-444	1	.....										
4	978	0.007	19.09	4.64E-79	236-269	0	.....											
K/LHDEIQELQAQIQEQHVQIDVDVSKPDLTAALR/D																		
599	Lamin-A	LMNA	P48679	LC-ESI-IT	163.5	21	16	28%		R/ETNLESPLVDTHSKR/T	3	612.93	0.725	14.26	1.32E-43	424-439	1	.....
										R/LLODSVDFSLADAINTEK/N	2	1063.5	0.033	14	2.45E-42	78-96	0	.....
										R/ISLPLPNFSSNLR/E	2	785.94	0.012	12.22	7.17E-32	410-423	0	.....
										R/TNKEVELOELNDR/F	3	529.93	0.007	11.57	2.41E-28	100-112	1	.....
										K/FADLSEANRN	2	547.25	0.022	11.49	4.35E-28	294-303	0	.....
										R/KVESLOEEAFKK/L	3	554.63	0.023	11.14	3.53E-26	222-235	2	.....
										R/LLODSVDFSLADAINTEK/N	2	1064.5	-1.01	10.49	1.53E-23	78-96	0	.....
										R/LLODSVDFSLADAINTEK/N	3	709.36	0.004	10.32	1.62E-22	78-96	0	.....
										Y/SSSPGGAYTR/S	2	541.27	-0.006	9.97	5.52E-21	53-63	half	.....
										R/ISLPLPNFSSNLR/E	2	786.48	-0.528	9.66	1.24E-19	410-423	0	.....
										K/ILLAEOLKGGK/S	3	513.98	-0.001	9.09	3.83E-17	129-142	1	.....
										K/MALDEIATY/K	2	655.3	1.03	9.04	4.53E-17	390-400	0	.....:Oxidation M:.....
										K/ILLAEOLK/G	2	585.35	0.011	8.28	3.67E-14	129-138	0	.....
										R/KVESLOEEAFKK/K	2	767.41	0.025	8.27	3.81E-14	222-234	1	.....
										S/PGGAYTR/S	2	410.72	-0.004	7.52	2.02E-11	56-63	half	.....
										S/SSPGGAYTR/S	2	497.76	-0.008	7.32	7.31E-11	54-63	half	.....
										R/IRLYREYDOLNIVK/M	3	561.3	0.685	6.33	8.92E-08	377-389	half	.....
										R/RLINATGEEVAMR/K	2	746.5	-0.133	16.04	1.47E-55	528-541	0	.....:Oxidation M:.....
										R/ALINATGEEVAMR/K	2	739.47	-1.09	16	3.04E-55	528-541	0	.....
										R/NSNLVGAHEELOSR/I	2	877.04	-0.105	15.49	8.30E-52	281-296	0	.....
R/ALINATGEEVAMR/K	2	738.49	-0.11	15.24	4.25E-50	528-541	0	.....										
603	Lamin-A	LMNA	P48679	LC-ESI-IT	446.7	90	48	50%		R/SGAASSTPLSPTR/I	2	680.55	-0.203	13.29	6.05E-38	Dec-25	0	.....
										R/SVGGSGGSGDNLVTR/S	2	783.99	-0.111	12.77	5.23E-35	629-645	0	.....
										R/MOOLDEYOELDIK/L	2	955.62	-0.156	11.57	2.54E-28	352-366	0	.....:Oxidation M:.....
										R/SLEENAGRL	2	545.39	-0.11	11.31	3.21E-27	51-60	0	.....
										R/MOOLDEYOELDIK/L	2	947.62	-0.153	11.12	1.92E-26	352-366	0	.....
										R/DLSAOLSOLOK/O	2	716.11	-0.214	10.16	1.76E-21	299-311	0	.....
										K/AYAEALGDAR/K	2	583.39	-0.112	9.74	4.47E-20	79-89	0	.....
										R/GSHCSSGGDPAEYILR/S	3	579.74	-0.159	9.44	2.88E-18	569-584	0	.....:Oys CAM:.....
										R/LADALDEL/A	2	514.42	0.37	8.84	2.43E-16	241-249	0	.....
										R/LOTKEELDFOK/N	2	746.52	-0.114	8.62	1.57E-15	197-208	1	.....
										R/LRDELSAR/E	2	594.47	-0.147	8.57	2.41E-15	320-329	1	.....
										R/SLTMVEDNDEEDGDELLH/H	3	814.84	-0.172	7.9	5.35E-13	546-566	half	.....
										R/LADALDEL/A	2	515.27	-0.48	7.75	7.45E-12	241-249	0	.....
										R/LOLESK/V	2	416	-0.247	7.52	1.54E-11	102-108	0	.....
										R/LOTKEELDFOK/N	3	498.22	-0.28	7.46	2.68E-11	197-208	1	.....
										G/AAHEELOSR/I	2	585	-0.211	6.19	1.20E-07	287-296	half	.....
R/SVGGSGGSGDNLVTR	2	705.92	-0.092	6.04	3.47E-07	629-644	half	.....										
R/ALINATGEEVAMR/K																		
599	Lamin-A	LMNA	P48679	LC-ESI-Q-TOF	no					R/ALINATGEEVAMR/K	2	738.4	-0.02	14.09	1.17E-47	528-541	0	.....:Oxidation M:.....
										R/ALINATGEEVAMR/K	2	746.42	-0.043	13.72	1.68E-40	528-541	0	.....
										R/SGAASSTPLSPTR/I	2	680.37	-0.203	12.49	1.98E-33	Dec-25	0	.....
										R/NSNLVGAHEELOSR/I	2	877.01	-0.075	12.33	1.10E-32	281-296	0	.....
603	Lamin-A	LMNA	P48679	LC-ESI-IT	446.7	90	48	50%		R/LOEKEDLOELNDR/L	2	815.45	-0.042	12.25	3.94E-32	29-41	1	.....
										R/MOOLDEYOELDIK/L	2	955.57	-0.106	11.93	1.23E-30	352-366	0	.....:Oxidation M:.....
																	.....	
																	.....	













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